

(19)



(11)

**EP 2 274 331 B1**

(12)

**EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:  
**06.11.2013 Bulletin 2013/45**

(51) Int Cl.:  
**C07K 14/78 (2006.01) C07K 16/00 (2006.01)**

(21) Application number: **09738252.7**

(86) International application number:  
**PCT/EP2009/055365**

(22) Date of filing: **04.05.2009**

(87) International publication number:  
**WO 2009/133208 (05.11.2009 Gazette 2009/45)**

(54) **IMPROVED FIBRONECTIN-BASED BINDING MOLECULES AND USES THEREOF**

VERBESSERTE BINDEMÖLEKÜLE AUF FIBRONECTIN-BASIS UND IHRE VERWENDUNG

MOLÉCULES DE LIAISON À BASE DE FIBRONECTINE AMÉLIORÉES ET LEURS UTILISATIONS

(84) Designated Contracting States:  
**AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO SE SI SK TR**

(56) References cited:  
**WO-A-02/04523 WO-A-02/32925  
WO-A-03/104418**

(30) Priority: **02.05.2008 US 50142 P**

(43) Date of publication of application:  
**19.01.2011 Bulletin 2011/03**

(60) Divisional application:  
**11153773.4 / 2 383 292  
11191379.4 / 2 439 212**

(73) Proprietor: **Novartis AG  
4056 Basel (CH)**

(72) Inventors:  
• **HASTEWELL, John  
Massachusetts 02139 (US)**  
• **LOEW, Andreas  
Massachusetts 02139 (US)**

(74) Representative: **Badur, Ralf et al  
Novartis Pharma AG  
Patent Department  
Forum 1  
Novartis Campus  
4056 Basel (CH)**

- **BATORI V ET AL: "Exploring the potential of the monobody scaffold: effects of loop elongation on the stability of a fibronectin type III domain" PROTEIN ENGINEERING, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 15, no. 12, 1 January 2002 (2002-01-01), pages 1015-1020, XP002996047 ISSN: 0269-2139**
- **KOIDE AKIKO ET AL: "Probing protein conformational changes in living cells by using designer binding proteins: Application to the estrogen receptor" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 99, no. 3, 5 February 2002 (2002-02-05), pages 1253-1258, XP009123728 ISSN: 0027-8424**
- **LIPOVSEK ET AL: "Evolution of an Interloop Disulfide Bond in High-Affinity Antibody Mimics Based on Fibronectin Type III Domain and Selected by Yeast Surface Display: Molecular Convergence with Single-Domain Camelid and Shark Antibodies" JOURNAL OF MOLECULAR BIOLOGY, LONDON, GB, vol. 368, no. 4, 17 April 2007 (2007-04-17), pages 1024-1041, XP022030013 ISSN: 0022-2836**

Note: Within nine months of the publication of the mention of the grant of the European patent in the European Patent Bulletin, any person may give notice to the European Patent Office of opposition to that patent, in accordance with the Implementing Regulations. Notice of opposition shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

**EP 2 274 331 B1**

**Description**Priority Information

5 **[0001]** This application claims priority to U.S. Provisional Patent Application Ser. No. 61/050,142, filed May 2, 2008, the contents of which are incorporated herein by reference in their entirety.

Related Information

10 **[0002]** The contents of any patents, patent applications, and references cited throughout this specification are hereby incorporated by reference in their entireties.

Background of the Invention

15 **[0003]** Molecules capable of specific binding to a desired target epitope are of enormous importance as both therapeutics and medical diagnostic tools. A well known example of this class of molecules is the monoclonal antibody. Antibodies can be selected that bind specifically and with high affinity to almost any structural epitope. As a result, antibodies are used routinely as research tools and as FDA approved therapeutics such that the worldwide market for therapeutic and diagnostic monoclonal antibodies is currently worth approximately \$30 billion.

20 **[0004]** However, monoclonal antibodies have a number of shortcomings. For example, classical antibodies are large and complex molecules. They have a heterotetrameric structure comprising two light chains and two heavy chains connected together by both inter and intra disulphide linkages. This structural complexity precludes easy expression of antibodies in simple prokaryotic systems and requires that antibodies are produced in more elaborate (and expensive) mammalian cell systems. The large size of antibodies also limits their therapeutic effectiveness since they are often  
25 unable to efficiently penetrate certain tissue spaces. Therapeutic antibodies, because they possess an Fc region, occasionally trigger undesired effector cell function and/or clotting cascades. In addition, generating bispecific or multispecific antibodies often involves difficult and complex procedures (See e.g., Josefine et al., (1997), Nature Biotechnology, 15: 159-163; and Wu et al. (2007) Nature Biotechnology, 25: 1290-1297).

30 **[0005]** Accordingly there is a need in the art for alternative binding molecules capable of specific binding to a desired target with high affinity and specificity. A need also exists for a simple method to generate bispecific or multispecific binding molecules.

Summary of the Invention

35 **[0006]** The present invention provides fibronectin type III (Fn3)-based binding molecules that specifically bind to a target antigen and, thus, can be used in a broad variety of therapeutic and diagnostic applications. The invention is based on the discovery of novel bispecific Fn3-based binding molecules in which a single Fn3 molecule can bind one or more target molecules by using both the top and bottom loops of Fn3. The disclosure also provides a simple, efficient method for generating bispecific and multispecific Fn3-based binding molecules.

40 **[0007]** The disclosure pertains to monospecific Fn3-based binding molecules that use the bottom AB, CD, EF loops or C-terminal to bind one or more target(s) ("bottom monospecific Fn3-based binding molecules") e.g., Human Serum Albumin (HSA), lysozyme. In one embodiment of the invention, these bottom monospecific Fn3-based binding molecules can be linked together (e.g., in pearl-like fashion) to form multispecific Fn3-based binding molecules that simultaneously bind to multiple targets, and/or can be conjugated to one or more non-Fn3 moieties (e.g., functional moieties), such as  
45 Human Serum Albumin (HSA), an antibody Fc region or polyethylene glycol (PEG), for example, to improve half life and stability of the Fn3-based binding molecule.

**[0008]** In another, the bottom monospecific Fn3-based binding molecules can be combined with Fn3-based binding molecules that use the top BC, DE, or FG loops to produce bispecific Fn3-based binding molecules. The invention disclosure further provides bispecific Fn3-based binding molecules that bind to two or more targets simultaneously.  
50 These bispecific Fn3-based binding molecules also can be linked together (e.g., in pearl-like fashion) to form multispecific Fn3-based binding molecules that simultaneously bind to multiple targets, and/or can be conjugated to one or more non-Fn3 moieties (e.g., functional moieties) as described above. The disclosure further provides methods of screening libraries of Fn3-based binding molecules for specific binding to a target, typically a target protein, as well as methods for manufacturing Fn3-based binding molecules in, for example, prokaryotic or eukaryotic systems. Still further, the disclosure provides compositions (e.g., therapeutic compositions) comprising Fn3-based binding molecules, and uses  
55 of such compositions in a variety of therapeutic and diagnostic applications.

**[0009]** Accordingly, in one aspect, the disclosure provides an Fn3-based binding molecule comprising an Fn3-domain, wherein at least one amino acid in one or more of the bottom AB, CD, EF loop regions or the C-terminus of the Fn3

domain are altered compared to a wild-type Fn3 domain (e.g., SEQ ID NO:1) to create a non-Fn3 binding sequence which binds to a specific target. In a particular disclosure, the altered amino acid residues in the AB, CD, EF loop regions or C-terminus includes one or more of the amino acids at position 15, 16, 38, 39, 40, 41, 42, 43, 44, 45, 60, 61, 62, 63, 64, 93, 95, or 96 of SEQ ID NO:1. The non-Fn3 binding sequence can be, for example, all or a portion of a CDR region (e.g., an antibody CDR region) or a T-cell receptor.

**[0010]** In another aspect, the disclosure pertains to a Fn3-based binding molecule comprising a first Fn3-domain, wherein at least one amino acid in one or more of the bottom AB, CD or EF loop regions or C-terminal of the Fn3 domain are altered compared to the wild-type Fn3 domain comprising SEQ ID NO: to create a non-Fn3 binding sequence which binds to a first target, and wherein at least one amino acid in one or more of the bottom AB, CD or EF loop regions or C-terminal of a second Fn3 domain are altered compared to the wild-type Fn3 domain comprising SEQ ID NO: to create a non-Fn3 binding sequence which binds to a second target.

**[0011]** In one aspect, the invention provides a bispecific Fn3-based binding molecule comprising an Fn3 domain, wherein at least one amino acid in one or more of the bottom AB, CD, EF loop regions or C-terminus of the Fn3 domain are altered compared to a wild-type Fn3 domain (e.g., SEQ ID NO:1) to create a non-Fn3 binding sequence which binds to a first target, and wherein at least one amino acid in one or more of the top BC, DE or FG loop regions of the Fn3 domain are altered compared to a wild-type Fn3 domain comprising (e.g., SEQ ID NO:1) to create a non-Fn3 binding sequence which binds to a second target. In a particular embodiment, the altered amino acid residues in the bottom AB, CD, EF loop regions or C-terminus includes one or more of the amino acids at position 15, 16, 38, 39, 40, 41, 42, 43, 44, 45, 60, 61, 62, 63, 64, 93, 95, or 96 of SEQ ID NO: 1, and the altered the amino acid residues in the top BC, DE or FG loop regions includes one or more of the amino acids at position 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 51, 52, 53, 54, 55, 56, 76, 77, 78, 79, 80, 80, 81, 82, 83, 84, 85, 86, 87, or 88 of SEQ ID NO: 1. Accordingly, such bispecific Fn3-based binding molecules of the invention simultaneously bind to two or more targets present on the same molecule, or on different molecules.

**[0012]** In another aspect, the invention provides, multispecific Fn3-based binding molecules comprising two or more Fn3-based binding molecules of any of the above described molecules, linked together (e.g., in pearl-like fashion), such that they bind two or more targets on the same molecule or on different molecules.

**[0013]** "Fn3-based binding molecules" of the invention, (which hereafter, include bispecific and multispecific Fn3-based binding molecules) also can bind to, or be linked to, one or more non-Fn3 moieties that, for example, increase the half-life of the Fn3-based binding molecule when administered *in vivo*. Suitable non-Fn3 moieties include, but are not limited to, antibody Fc regions, Human Serum Albumin (HSA) (or portions thereof), polyethylene glycol (PEG) and/or polypeptides which bind to the aforementioned proteins or other serum proteins with increased half-life, such as, e.g., transferrin. Accordingly, in another aspect, the invention provides conjugates which include one or more Fn3-based binding molecules linked to a non-Fn3 moiety.

**[0014]** In yet another embodiment, the Fn3-based binding molecules and conjugates of the invention further comprise at least one modified amino acid residue compared to a wild-type Fn3 domain (e.g., SEQ ID NO: 1) for attaching a functional moiety. In one embodiment, the modified amino acid comprises substitution or addition of a cysteine or non-natural amino acid residue in one or more regions selected from a loop region, a beta-strand region, a beta-like strand region, a C-terminal region, the region between the C-terminus and the most C-terminal beta strand (or beta-like strand), an N-terminal region, and the region between the N-terminus and the most N-terminal beta strand (or beta-like strand).

**[0015]** Fn3-based binding molecules of the invention can be based on wild-type Fn3 sequences, (e.g., human Fn3 having the amino acid sequence shown in SEQ ID NO: 1) as well as modified versions of such wild type sequences, as discussed herein. For example, the Fn3-based binding molecule can be a chimera having Fn3 beta-strands derived from at least two different fibronectin modules.

**[0016]** Also provided by the invention are compositions comprising the Fn-3 based binding molecules and conjugates of the invention, formulated with a suitable carrier.

**[0017]** The Fn-3 based binding molecules and conjugates of the invention can be used in a variety of therapeutic and diagnostic applications including, but not limited to, applications that antibodies can be used in. Such applications include, for example, treatment and diagnosis of autoimmune diseases, cancers and infectious diseases.

**[0018]** Other features and advantages of the invention will be apparent from the following detailed description and claims, such as a variegated nucleic acid library encoding Fn3-based binding molecules.

#### Brief Description of the Drawings

**[0019]**

*Figure 1* shows a ribbon diagram of a Fn3-based binding molecule with loop and framework residues constituting the ligand binding surfaces highlighted.

## EP 2 274 331 B1

Figure 2 depicts a schematic showing the top and bottom loop faces of Fn3.

Figure 3A is a schematic of a monospecific Fn3-binding molecule showing that either the top or bottom loops can be modified to bind to a single target, Target A.

Figure 3B is a schematic of a bispecific Fn3-based binding molecule showing that both the top and bottom loops can be modified to bind a single target, Target A. The bispecific Fn3-based binding molecule can also be used to bind to two different targets, where the top loop binds to one target, Target A and bottom loops bind to a second target, Target B.

Figure 4A is a schematic of a multispecific Fn3-based binding molecule comprising two bottom monospecific Fn3 domains with modifications to the bottom loops that are linked together.

Figure 4B is a schematic of a multispecific Fn3-based binding molecule comprising two bispecific Fn3 domains with modifications to the top and bottom loops that are linked together.

Figure 5A is a schematic of a multispecific Fn3-binding molecule comprising multiple monospecific Fn3 domains with modifications to the bottom loops that are linked together.

Figure 5B is a schematic of a multispecific Fn3-binding molecule comprising multiple bispecific Fn3 domains with modifications to the top and bottom loops that are linked together.

Figure 6 is a diagram of the vector used for cloning the fibronectin cDNA.

Figure 7 is a table showing the amino acid sequences of a selected number of monospecific Fn3-based binding molecules that use the bottom loops or C-terminal to bind human serum albumin.

Figure 8 is a table showing the amino acid sequences of a selected number of monospecific Fn3-based binding molecules that use the bottom loops or C-terminal to bind lysozyme.

Figure 9 is a graph showing human serum albumin specific ELISA analysis of His-tagged monospecific Fn3-based binding molecules that use the bottom loops or C-terminal to bind human serum albumin as the specific target.

Figure 10 is an SDS-PAGE gel showing successful expression in *E. coli* of monospecific Fn3-based binding molecules that use the bottom loops or C-terminal to bind a specific target.

Figure 11 is an SDS-PAGE gel showing successful purification from *E. coli* lysates of monospecific Fn3-based binding molecules that use the bottom loops or C-terminal to bind a specific target.

Figure 12 is an SDS-PAGE gel showing successful purification from *E. coli* lysates of bispecific Fn3-based binding molecules that use the bottom loops or C-terminal to bind a specific target.

Figure 13 is a graph showing ELISA analysis of bispecific Fn3-based binding molecules that use both the bottom loops or C-terminal and the top loops to bind different target molecules. Bispecific Fn3-based binding molecules isolated from clone 87 and clone 89 have top loops that bind VEGFR2 and bottom loops that bind human serum albumin. The data shows that a single Fn3-based binding molecule can be engineered such that the top loops bind to VEGFR2 and the bottom loops bind to HSA.

Figure 14 is a graph showing Biacore binding study of a bispecific Fn3-based binding molecule isolated from clone 87 that uses the top loops to bind VEGFR2.

Figure 15 is a graph showing Biacore binding study of a bispecific Fn3-based binding molecule isolated from clone 87 that uses the bottom loops or C-terminal to bind HSA.

### Detailed Description of the Invention

**[0020]** In order to provide a clear understanding of the specification and claims, the following definitions are conveniently provided below.

*Definitions*

**[0021]** As used herein, the term "Fibronectin type III domain" or "Fn3 domain" refers to a wild-type Fn3 domain from any organism, as well as chimeric Fn3 domains constructed from beta strands from two or more different Fn3 domains. As is known in the art, naturally occurring Fn3 domains have a beta-sandwich structure composed of seven beta-strands, referred to as A, B, C, D, E, F, and G, linked by six loops, referred to as AB, BC, CD, DE, EF, and FG loops (See e.g., Bork and Doolittle, Proc. Natl. Acad. Sci. U.S.A 89:8990, 1992; Bork et al., Nature Biotech. 15:553, 1997; Meinke et al., J. Bacteriol. 175:1910, 1993; Watanabe et al., J. Biol. Chem. 265:15659, 1990; Main *et al.*, 1992; Leahy *et al.*, 1992; Dickinson *et al.*, 1994; U.S. patent 6,673,901; Patent Cooperation Treaty publication WO/03104418; and, US patent application 2007/0082365, the entire teachings of which are incorporated herein by reference). Three loops are at the top of the domain (the BC, DE and FG loops) and three loops are at the bottom of the domain (the AB, CD and EF loops) (see Figure 1). In a particular embodiment, of the invention, the Fn3 domain is from the tenth Fn3 domain of human Fibronectin (<sup>10</sup>Fn3) (SEQ. ID. NO: 1).

**[0022]** As used herein the term "Fn3-based binding molecule" or "fibronectin type III (Fn3)-based binding molecule" refers to an Fn3 domain that has been altered to contain one or more non-Fn3 binding sequences. In a particular embodiment, one or more of the bottom AB, CD and/or EF loops are altered compared to the corresponding wild-type Fn3 domain to contain one or more non-Fn3-binding sequences. In another embodiment, one or more of the bottom AB, CD or EF loops and one or more of the top BC, DE and FG loops are altered compared to the corresponding wild-type Fn3 domain to contain one or more non-Fn3-binding sequences. Such molecules are referred to herein as "bispecific Fn3-based binding molecules". In a further embodiment, two or more Fn3-based binding molecules or bispecific Fn3-based binding molecule are linked together. Such molecules are referred to herein as "multispecific Fn3-based binding molecules".

**[0023]** The term "non-Fn3 binding sequence" refers to an amino acid sequence which is not present in the naturally occurring (e.g., wild-type) Fn3 domain, and which binds to a specific target. Such non-Fn3 binding sequences are typically introduced by modifying (e.g., by substitution and/or addition) the wild-type Fn3 domain (e.g., within the bottom loops and/or top loop regions). This can be achieved by, for example, random or predetermined mutation of amino acid residues within the wild-type Fn3 domain. Additionally or alternatively, the non-Fn3 binding sequence can be partly or entirely exogenous, that is, derived from a different genetic or amino acid source. For example, the exogenous sequence can be derived from a hypervariable region of an antibody, such as one or more CDR regions having a known binding specificity for a known target antigen. Such CDRs can be derived from a single antibody chain (e.g. a variable region of a light or heavy chain) or from a combination of different antibody chains. The CDRs can also be derived from two different antibodies (e.g., having different specificities). In a particular embodiment, the CDR(s) are derived from a nanobody, for example, a Camelidae-like heavy chain.

**[0024]** The term "monospecific" as used herein refers to an Fn3-based binding molecule that binds to one or more target molecules comprising Fn3 domains in which only the bottom region of the Fn3 domain, or the top region of the Fn3 domain, but not both, are used for binding. For example, a bottom monospecific Fn3-based binding molecule is one that uses only the bottom loops, such as the AB, CD, or EF loops, or C-terminal of the Fn3 domain to bind a target, while a top monospecific Fn3-based binding molecule uses only the top loops of the Fn3 domain, such as BC, DE, and FG loops, to bind the target. It is to be understood that not all three loops from the top or bottom region need to be used for binding the target molecule.

**[0025]** The monospecific Fn3 domains can also be linked together (e.g., in a pearl-like fashion) to form a multispecific Fn3-based binding molecules that comprises, for example, at least two monospecific Fn3 domains that are linked together. For bottom monospecific binding molecules, each of the Fn3 domains uses at least one bottom loop or C-terminal region to bind to one or more target molecules. In one embodiment, this multispecific Fn3-based binding molecule binds to different target regions of a same target molecule (e.g., Target A). For example, one Fn3 domain can bind to a first target region of Target A and another Fn3 domain can bind to a second target region of Target A. This can be used to increase avidity of the Fn3-based binding molecule for the target molecule. In another embodiment, the multispecific Fn3-based binding molecule binds to multiple target molecules. For example, one Fn3 domain can bind to Target A and another Fn3 domain can bind to Target B (e.g., a half life extender). In yet another embodiment, the multispecific Fn3-based binding molecule comprises at least two monospecific Fn3 domains that bind to different target regions of Target A and at least two monospecific Fn3 domains that bind to different target regions of Target B. The skilled artisan will appreciate that any number of Fn3 domains can be linked in this fashion to create a multispecific Fn3-based binding molecule that are able to bind to different target regions of the same target molecule or different target molecules.

**[0026]** The term "bispecific" as used herein refers to an Fn3-based binding molecule that binds to one or more targets using both the bottom region of the Fn3 domain and the top region of the Fn3 domain. For example a bispecific Fn3-based binding molecule comprises Fn3 domains that use both the bottom loops, such as the AB, CD, or EF loops, or C-terminal of the molecule and the top loops of the molecule, such as BC, DE, and FG loops, to bind the target. The bispecific Fn3-based binding molecules can be used to bind the same target molecule, e.g., Target A, which can bind

to both the top and bottom of the bispecific Fn3-based binding molecule (See Figure 3b). Alternatively, the bispecific Fn3-based binding molecule can be used to bind to two different target molecules, e.g., Target A and Target B. In this instance, the top loops can be used to bind to Target A and the bottom loops can be used to bind to Target B, or *visa versa* (See Figure 3b). The bispecific Fn3-based binding molecules can also be linked together (e.g., in a pearl-like fashion) to form a multispecific Fn3-based binding molecules.

**[0027]** The term "multispecific" as used herein refers to a Fn3-based binding molecule that comprises at least two monospecific Fn3-based binding molecules linked together or at least two bispecific Fn3-based binding molecules linked together.

**[0028]** The term "complementarity determining region (CDR)" refers to a hypervariable loop from an antibody variable domain or from a T-cell receptor. The position of CDRs within an antibody variable region have been precisely defined (see, Kabat, E.A., et al. Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, 1991, and Chothia, C. et al., J. Mol. Biol. 196:901-917, 1987, which are incorporated herein by reference).

The term "single domain antibodies" refers to any naturally-occurring single variable domain and corresponding engineered binding fragments, including human domain antibodies as described by Domantis (Domantis / GSK (Cambridge, UK) or camelid nanobodies as defined hereafter.

**[0029]** The term "single chain antibody" refers to an antigen binding portion of a light chain variable region and an antigen binding portion of a heavy chain variable region, joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see *e.g.*, Bird et al. (1988) Science 242:423-426; and Huston et al. (1988) Proc. Natl. Acad. Sci. U.S.A 85:5879-5883).

**[0030]** The term "camelid nanobody" refers to a region of camelid antibody which is the small single variable domain devoid of light chain and that can be obtained by genetic engineering to yield a small protein having high affinity for a target, resulting in a low molecular weight antibody-derived protein. See WO07042289 and U.S. patent number 5,759,808 issued June 2, 1998; see also Stijlemans, B. et al., 2004. Engineered libraries of camelid antibodies and antibody fragments are commercially available, for example, from Ablynx, Ghent, Belgium. As with other antibodies of non-human origin, an amino acid sequence of a camelid antibody can be altered recombinantly to obtain a sequence that more closely resembles a human sequence, *i.e.*, the nanobody can be "humanized". Thus the natural low antigenicity of camelid antibodies to humans can be further reduced.

**[0031]** The term "target" refers to an antigen or epitope recognized by Fn3-based binding molecule of the invention. Targets include, but are not limited to, epitopes present on proteins, peptides, carbohydrates, and/or lipids.

**[0032]** The term "conjugate" refers to an Fn3-based binding molecule chemically or genetically linked to one or more non-Fn3 moieties.

**[0033]** The term "non-Fn3 moiety" refers to a biological or chemical entity that imparts additional functionality to a molecule to which it is attached. In a particular embodiment, the non-Fn3 moiety is a polypeptide, *e.g.*, human serum albumin (HSA), or a chemical entity, *e.g.*, polyethylene glycol (PEG) which increases the half-life of the Fn3-based binding molecule *in vivo*.

**[0034]** The term "non-natural amino acid residue" refers to an amino acid residue that is not present in the naturally occurring (wild-type) Fn3 domain. Such non-natural amino acid residues can be introduced by substitution of naturally occurring amino acids, and/or by insertion of non-natural amino acids into the naturally occurring amino acid Fn3 sequence. The non-natural amino acid residue also can be incorporated such that a desired functionality is imparted to the Fn3-based binding molecule, for example, the ability to link a functional moiety (*e.g.*, PEG).

**[0035]** The term "polyethylene glycol" or "PEG" refers to a polyalkylene glycol compound or a derivative thereof, with or without coupling agents or derivatization with coupling or activating moieties.

**[0036]** The term "specific binding" or "specifically binds to" refers to the ability of an Fn3-based binding molecule to bind to a target with an affinity of at least  $1 \times 10^{-6}$  M, and/or bind to a target with an affinity that is at least two-fold, (preferably at least 10 fold), greater than its affinity for a nonspecific antigen at room temperature under standard physiological salt and pH conditions, as measured by surface plasmon resonance.

## Overview

**[0037]** The present invention provides fibronectin type III (Fn3)-based binding molecules that specifically bind to a target antigen. The disclosure provides for monospecific Fn3-binding molecules with modifications to at least one or more of the bottom AB, CD, EF loop regions or C-terminus that binds to one or more target molecules. The invention further provides bispecific Fn3-based binding molecules that bind to the same target molecule with two opposite binding sites or to two or more target antigens simultaneously. The Fn3-based binding molecules of the invention also can be linked together (*e.g.*, in pearl-like fashion) to form multispecific Fn3-based binding molecules and/or can be conjugated to a non-Fn3 moiety, such as Human Serum Albumin (HSA), to improve half life and stability. The Fn3-based binding

molecules can be used in a variety of therapeutic and diagnostic applications, much like other binding molecules, such as antibodies.

#### Monospecific Fn3-Based Binding Molecules

- 5
- [0038]** In one aspect, the disclosure provides monospecific Fn3-based binding molecules which are altered compared to the wild-type Fn3 domain (e.g., in the bottom and/or top loop regions) to create a non-Fn3 binding sequence which binds to a specific target. The disclosed monospecific Fn3-based binding molecules use the bottom AB, CD, EF loops or C-terminal to bind one or more targets molecules.
- 10 **[0039]** Accordingly, in one aspect, the disclosure provides an Fn3-based binding molecule comprising an Fn3 domain, wherein at least one amino acid in one or more of the of the Fn3 domain is altered compared to a wild-type Fn3 domain to create a non-Fn3 binding sequence which binds to a specific target. In particular, the wild type Fn3 domain is a human Fn3 domain, e.g., human <sup>10</sup>Fn3 (SEQ ID NO:1). Additionally, one or more amino acid residues in one or more beta-strands adjacent to the loop regions can also be mutated compared to the wild-type Fn3 domain to contribute additional
- 15 non-Fn3 binding sequences or residues which can be linked to non-Fn3 moieties. Particular amino acid residues in the bottom AB, CD, EF loop regions or C-terminus and adjacent beta strands which can be altered include, for example, amino acids at position 15, 16, 38, 39, 40, 41, 42, 43, 44, 45, 60, 61, 62, 63, 64, 93, 95, or 96 of SEQ ID NO:1.
- [0040]** Exemplary non-Fn3 binding sequences include, for example, all or a portion of a complementarity determining region (CDR) of an antibody or a T-cell receptor.
- 20 **[0041]** In another aspect, the invention provides a library of Fn3-based binding molecules, which can be used to identify Fn3-based binding molecules which bind to a particular desired target. In one embodiment, the library comprises Fn3-based binding molecules, each of which contains at least one amino acid alteration in one or more of the bottom AB, CD, EF loop regions or C-terminus compared to a wild-type Fn3 domain, such as the human <sup>10</sup>Fn3 (SEQ ID NO: 1). Particular amino acid residues in the AB, CD or EF loop regions and beta strands which can be altered include, for
- 25 example, amino acids at position 15, 16, 38, 39, 40, 41, 42, 43, 44, 45, 60, 61, 62, 63, 64, 93, 95, or 96 of SEQ ID NO:1.
- [0042]** In a another embodiment, the library comprises Fn3-based binding molecules, each of which contains at least one amino acid alteration in one or more of the top BC, DE or FG loop regions compared to a wild-type Fn3 domain, such as the human <sup>10</sup>Fn3 (SEQ ID NO: 1). Particular amino acid residues in the BC, DE or FG loop regions and beta strands which can be altered include, for example, amino acids at position 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 51, 52,
- 30 53, 54, 55, 56, 76, 77, 78, 79, 80, 80, 81, 82, 83, 84, 85, 86, 87, or 88 of SEQ ID NO:1.
- [0043]** Library diversity can be generated by, for example, random mutagenesis, "walk through mutagenesis, or "look through mutagenesis of one or more of the disclosed residues in SEQ ID NO:1 (7,195,880; 6,951,725; 7,078,197; 7,022,479; 5,922,545; 5,830,721; 5,605,793, 5,830,650; 6,194,550; 6,699,658; 7,063,943; 5,866,344 and Patent Cooperation Treaty publication WO06023144).
- 35 **[0044]** Alternatively, Fn3-based binding molecules can be generated by combining non-Fn3-binding sequences from one or more different library members. In a particular embodiment, bispecific Fn3-based binding molecules are generated by combining non-Fn3-binding sequences from one or more of the bottom AB, CD, EF loop regions or C-terminus of one library member, together with non-Fn3-binding sequences from one or more of the top BC, DE or FG loop regions of another library member, into a single bispecific Fn3-based binding molecule.
- 40 **[0045]** Nucleic acids encoding the library of Fn3-based binding molecules, or variants thereof, described herein can be constructed using art recognized methods including, but not limited to, PCR-based or enzyme-mediate genetic engineering, *ab initio* DNA or RNA synthesis, and/or cassette mutagenesis.
- [0046]** Suitable targets for Fn3-based binding molecules include, but are not limited to, half-life extenders (e.g., HSA), lysozyme, a cellular receptor, a cellular receptor ligand, a bacteria, or a virus (see, Figure 1). In a particular embodiment the target is involved in a human disease, e.g., an autoimmune disease, cancer, or an infectious disease.
- 45 **[0047]** This specification describes, inter alia, the identification and production of novel, Fn3-based binding molecules that bind to a target, e.g., HSA, using the bottom loops of the Fn3 molecule. Hundreds of clones were generated using the methods of the invention and a representative few have been characterized as shown in Example 4. Figure 7 shows a representative number of sequences isolated from this library of Fn3 scaffold proteins that bind to HSA (SEQ ID NOs: 7-41).
- 50 **[0048]** Furthermore, it was discovered that many independently randomized loops tended to converge to a consensus sequence that is likely to participate in HSA binding (SEQ ID NO: 42). Therefore, it is expected that polypeptides having this consensus sequences will be useful as HSA binding agents even when separated from the protein context in which they were identified.
- 55 **[0049]** Figure 8 shows a representative number of sequences isolated from this library of Fn3 scaffold proteins that bind to lysozyme (SEQ ID NOs: 43-116). The data in the Example 5 shows binding studies of s representative few Fn3-based binding proteins for HSA. The results show that Fn3 binding molecules can be generated with modified bottom loops that have the ability to bind a target.

[0050] This data shows that a library of Fn3 -based binding molecules can be generated in which at least one of the bottom loops is modified such that it binds to a target. With the HSA library, the bottom loops were kept at the same length as wild type Fn3. With the lysozyme library, the bottom loops were varied in length without adverse effects on the protein structure. Furthermore, these monospecific Fn3 - based binding molecules with the modified bottom loops, maintain conformational stability and can be expressed and purified while retaining binding ability. Thus, the methods of the disclosure can be used to generate a library of Fn3-based binding molecules that have at least one modified bottom loop as well as Fn3-based binding molecules that use the bottom face to bind a target.

#### Bispecific Fn3-Based Binding Molecules

[0051] As discussed in part above, in another aspect, the invention provides bispecific Fn3-based binding molecules which are able to bind to two or more targets simultaneously. Such bispecific Fn3-based binding molecules comprise two or more non-Fn3 binding sequences within the same protein domain. This can be achieved by altering any two or more of the top and bottom AB, BC, CD, DE, EF or FG loop regions to comprise non-Fn3 binding sequences (see Figure 1). In a particular embodiment, the bispecific Fn3-based binding molecule is altered in both the top and bottom loop regions to create two separate binding specificities.

[0052] Accordingly, in one embodiment, the invention provides a bispecific Fn3-based binding molecule comprising an Fn3 domain, wherein at least one amino acid in one or more of the bottom AB, CD or EF loop regions of the Fn3 domain is altered compared to a wild-type Fn3 domain (e.g., SEQ ID NO:1) to create a non-Fn3 binding sequence which binds to a first target, and wherein at least one amino acid in one or more of the top BC, DE and FG loop regions of the Fn3 domain is altered compared to a wild-type Fn3 domain (e.g., SEQ ID NO:1) to create a non-Fn3 binding sequence which binds to a second target. Particular amino acid residues in the bottom AB, CD, EF loop regions or C-terminus and adjacent beta strands which can be altered include, for example, amino acids at position 15, 16, 38, 39, 40, 41, 42, 43, 44, 45, 60, 61, 62, 63, 64, 93, 95, or 96 of SEQ ID NO: 1. Particular amino acid residues in the top BC, DE and FG loop regions and adjacent beta strands which can be altered include, for example, amino acids at position 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 51, 52, 53, 54, 55, 56, 76, 77, 78, 79, 80, 80, 81, 82, 83, 84, 85, 86, 87, or 88 of SEQ ID NO:1.

[0053] Bispecific Fn3-based binding molecules of the invention bind to two or more targets simultaneously. The targets can be present on the same molecule, such that two regions of the same target molecule become juxtaposed by the binding of the bispecific Fn3-based binding domain to the targets. Alternatively, the targets can be present on different molecules, such that the two different molecules become juxtaposed by the binding of the bispecific Fn3-based binding molecule to the targets. The targets can also be identical, such that the bispecific Fn3-based binding molecule is able to cluster target molecules, in the same way as an antibody is able to cluster molecules.

[0054] Suitable targets include those previously mentioned above, alone or in combination with targets present on molecules that enhance the physiochemical properties of the bispecific Fn3-based binding molecule, such as a half-life extender, e.g., HSA, an antibody Fc receptor or PEG. Suitable targets also include molecules that confer additional functional properties e.g. cytotoxic, labeling or imaging moieties, as described herein. Suitable targets can also be used to induce dimerization of bispecific Fn3-based binding molecules if the two bispecific Fn3-based binding domain molecules are capable of binding to the target simultaneously. For example, two bispecific Fn3-based binding molecules can work with an idiotype/anti-idiotype relationship. In this instance, one binding site of a Fn3-binding molecule will bind to a target molecule while the second binding site of the Fn3-binding molecule will bind to a binding region on the Fn3-binding molecule. For example, the bottom loops of the bispecific Fn3-binding molecule may bind to Target A while the top loops of the bispecific molecule may bind to a second bispecific Fn3-binding molecule. This will lead to multimerisation of the Fn3-binding molecules in such a fashion that the bottom loop target binding sites are available for binding Target A while the top loop binding sites lead to self association with a second Fn3-binding molecule.

[0055] This specification describes, inter alia, the identification and production of novel, bispecific Fn3 molecules that bind to two different target molecules, e.g., HSA and VEGFR2, using both the bottom loops and top loops of the same Fn3 molecule. Hundreds of clones have been generated using the methods of the invention and one in particular, clone 89 is described in more detail in Example 6 and 7. Clone 89 is a bispecific Fn3-based binding molecule in which the bottom loops of the Fn3 molecule bind to HSA while the top loops of the same Fn3 molecule bind to VEGFR2 (SEQ ID NO: 120).

[0056] Bispecific Fn3 binding molecules can also be created by first identifying a monospecific Fn3 -based binding molecule with a desired function, e.g., binding to HSA. This monospecific Fn3-based molecule only has loop variations in one of the two binding interfaces (i.e., either the top loops, or the bottom loops, but not both). Once a suitable monospecific Fn3-based binding molecule with the desired function is identified, this molecule is then used as a scaffold to generate a library in which the loops of the opposite face of the Fn3 molecule are varied, thus generating a library of bispecific Fn3-based binding molecules. This library of bispecific Fn3-based binding molecules is then used to screen against the second target molecule to identify a bispecific entity that binds to two targets. For example, the monospecific Fn3-based binding molecule can bind to HSA using at least one bottom loop. This monospecific Fn3-based binding

molecule is used as a scaffold to generate a library in which at least one of the top loops of the molecule is varied such that it binds to a second target, e.g., VEGFR2, thereby creating a library of bispecific Fn3-based molecules. This library of bispecific Fn3-based molecules can be used to screen for the VEGFR2 target.

**[0057]** VEGFR2 is the primary mediator for the proangiogenic effects of VEGF signaling. VEGFR-2 is bound and activated by VEGF-A, VEGF-C and VEGF-D. In endothelial cells, VEGFR-2 activation stimulates cell proliferation and migration, and in vivo, VEGFR-2 activation triggers angiogenesis and increases the permeability of the vasculature. Increased angiogenesis is well established as an important feature of tumor growth and various retinopathies, while increased permeability of the vasculature is a significant event in many inflammatory responses (See e.g., WO2005056764).

**[0058]** This data shows that the bispecific Fn3 - based binding molecules with the modified bottom loops, maintain conformational stability and can be expressed and purified while retaining binding ability. The skilled artisan will appreciate that any bispecific Fn3-based binding molecule can be generated using the methods of the invention disclosed herein. The bispecific molecule can be designed to bind to any target of interest. For example, the bispecific molecule can bind to different binding sites of the same target. Alternatively, the bispecific molecule can bind to different target molecules.

#### Multispecific Fn3-Based Binding Molecules

**[0059]** In another aspect, the invention provides multispecific Fn3-based binding molecules which comprise two or more monospecific Fn3-based binding molecules or bispecific Fn3-based binding molecules linked together (e.g., genetically or chemically). The multispecific Fn3-based binding molecules comprise at least one monomeric Fn3-based binding molecule that uses at least one of the bottom loops AB, CD, EF to bind to a target.

**[0060]** In one embodiment, the multispecific Fn3-based binding molecule comprises two or more Fn3-based binding molecules linked, in pearl-like fashion, wherein each individual Fn3-based binding molecule binds to a specific target. As with bispecific Fn3-based binding molecules, such targets can be present on the same molecule or on different molecules, such that the different molecules become juxtaposed by the binding of the multispecific Fn3-based binding molecule. The targets can also be identical, such that the multispecific Fn3-based binding molecule is able to cluster target molecules, in a similar way to an antibody. Avidity is also increased by binding to the same target molecule with two binding sites on the Fn3-based binding molecule capable of independently binding to different regions of the target molecule.

**[0061]** A variety of Fn3-based binding molecules can be incorporated into multispecific Fn3-based binding molecules, including Fn3-based binding molecules with a single binding specificity, bispecific Fn3-based binding molecules, or a combination of both types of Fn3-based binding molecules. Accordingly, suitable targets for multispecific Fn3-based binding molecules include those described above for Fn3-based binding molecules and bispecific Fn3-based binding molecules.

**[0062]** Multispecific Fn3-based binding molecules can be produced using art recognized methods. For example, Fn3-based binding molecules may be linked genetically, such that multispecific Fn3-based binding molecules are expressed as a single polypeptide. This linkage may be direct or conferred by an additional amino acid "linker" sequence. Suitable non-limiting methods and linkers are described, for example, in US20060286603 and WO04041862A2. Exemplary polypeptide linkers include, but are not limited to, GS linkers, such as GGGSGGGGS (SEQ ID NO: 118), GSGSGSGSGS (SEQ ID NO: 121), PSTSTST (SEQ ID NO: 122), and EIDKPSQ (SEQ ID NO: 123), and multimers thereof. Example 6 shows how to make a multispecific Fn3-based binding molecule using a G-S linker. Specifically, the multispecific Fn3-based binding molecule is created using the GGGSGGGGS (SEQ ID NO: 118) linker sequence that links a first monospecific Fn3-based binding molecule which binds VEGFR2 using the top loops with a second monospecific Fn3-based binding molecule which binds HSA using the bottom loops (SEQ ID NO: 119).

**[0063]** The multispecific Fn3-based binding molecules generated using linker sequences have an improved steric hinderance for binding to target molecules, thus enabling shorter linker sequences to be used to link two or more monomeric Fn3-based binding molecules together. Shorter linker sequences cause less immunogenic responses and are less likely to get cleaved.

**[0064]** Alternatively, multispecific Fn3-based binding molecules can be prepared by chemically conjugating the individual Fn3-based binding molecules using methods known in the art. A variety of coupling or cross-linking agents can be used for covalent conjugation. Examples of cross-linking agents include, e.g., protein A, carbodiimide, N-succinimidyl-S-acetyl-thioacetate (SATA), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), o-phenylenedimaleimide (oPDM), N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), and sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) (see e.g., Karpovsky et al. (1984) J. Exp. Med. 160:1686; Liu, MA et al. (1985) Proc. Natl. Acad. Sci. U.S.A 82:8648). Other methods include those described in Paulus (1985) Behring Ins. Mitt. No. 78, 118-132; Brennan et al. (1985) Science 229:81-83), and Glennie et al. (1987) J. Immunol. 139: 2367-2375). Preferred conjugating agents are SATA and sulfo-SMCC, both available from Pierce Chemical Co. (Rockford, IL). Cysteine residues can be introduced into the Fn3-binding molecules at specific positions (See e.g.,) and then crosslink with reagents to sulfhydryl such as

DPDPB or DTME (available from Pierce) to link two molecules together.

**[0065]** Examples 6 and 7 describe the identification and production of novel, bispecific Fn3 molecules that bind to two different targets, HSA and VEGFR2, using two separate monospecific Fn3 molecules that are linked together with a linker sequence. In this particular Example, one monomeric Fn3-based binding molecule uses the bottom loops to bind to HSA while the other monospecific Fn3-based binding molecule uses the top loops to bind VEGFR2. Hundreds of clones have been generated using the methods of the invention and a representative few have characterized, in particular clone 89. This data shows that the multispecific Fn3 - based binding molecules with the modified bottom loops, maintain conformational stability and can be expressed and purified while retaining binding ability. The skilled artisan will appreciate that any number of multispecific Fn3-based binding molecules can be generated using the methods of the invention. Such multispecific binding molecules can be designed to bind to a single target. Alternatively, the multispecific binding molecules can be designed to bind to two or more targets.

#### Conjugates of Fn3-Based Binding Molecules

**[0066]** In another aspect the invention provides conjugates comprising an Fn3-based binding molecule linked to one or more non-Fn3 moieties. Such non-Fn3 moieties can, for example, impart additional functional or physiochemical properties to the Fn3-based binding molecule. In one embodiment, the Fn3-based binding molecule is linked or fused to an antibody Fc domain (or a portion thereof). Methods for fusing molecules to Fc domains, e.g., the Fc domain of IgG1, are known in the art (see, e.g., U.S. 5,428,130). Such conjugates have increased circulating half-lives, due to the ability of Fc to bind to FcRn, which serves a critical function in IgG homeostasis, protecting molecules bound to it from catabolism.

**[0067]** In another embodiment, Fn3-based binding molecule is fused to one or more human serum albumin (HSA) polypeptides, or a portion thereof. HSA, a protein of 585 amino acids in its mature form, is responsible for a significant proportion of the osmotic pressure of serum and also functions as a carrier of endogenous and exogenous ligands. The role of albumin as a carrier molecule and its inert nature are desirable properties for use as a carrier and transporter of polypeptides in vivo. The use of albumin as a component of an albumin fusion protein as a carrier for various proteins has been suggested in WO 93/15199, WO 93/15200, and EP 413 622. The use of N-terminal fragments of HSA for fusions to polypeptides has also been proposed (EP 399 666). Accordingly, by genetically or chemically fusing or conjugating the molecules of the present invention to albumin, or a fragment (portion) or variant of albumin or a molecule capable of binding HSA (an "anti-HSA binder") that is sufficient to stabilize the protein and/or its activity, the molecule is stabilized to extend the shelf-life, and/or to retain the molecule's activity for extended periods of time in solution, in vitro and/or in vivo.

**[0068]** Fusion of albumin to another protein may be achieved by genetic manipulation, such that the DNA coding for HSA, or a fragment thereof, is joined to the DNA coding for the protein. A suitable host is then transformed or transfected with the fused nucleotide sequences, so arranged on a suitable plasmid as to express a fusion polypeptide. The expression may be effected in vitro from, for example, prokaryotic or eukaryotic cells, or in vivo e.g. from a transgenic organism. Additional methods pertaining to HSA fusions can be found, for example, in WO 2001077137 and WO 200306007, incorporated herein by reference. In a specific embodiment, the expression of the fusion protein is performed in mammalian cell lines, for example, CHO cell lines.

**[0069]** Other Fn3-based binding molecule conjugates of the present invention include an Fn3-based binding molecule linked to a non-Fn3-based binding molecule, e.g., another peptide or protein (e.g., an antibody or ligand for a receptor), to generate a molecule that binds to at least two different binding sites or target molecules.

**[0070]** The Fn3-based binding molecule conjugates of the present invention can be prepared by linking the constituent molecules using methods known in the art. For example, the constituent molecules can be chemically linked using a variety of coupling or cross-linking agents can be used for covalent conjugation. Examples of cross-linking agents include protein A, carbodiimide, N-succinimidyl-S-acetyl-thioacetate (SATA), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), o-phenylenedimaleimide (oPDM), N-succinimidyl-3-(2-pyridylthio)propionate (SPDP), and sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) (see e.g., Karpovsky et al. (1984) J. Exp. Med. 160:1686; Liu, MA et al. (1985) Proc. Natl. Acad. Sci. U.S.A 82:8648). Other methods include those described in Paulus (1985) Behring Ins. Mitt. No. 78, 118-132; Brennan et al. (1985) Science 229:81-83), and Glennie et al. (1987) J. Immunol. 139: 2367-2375). Preferred conjugating agents are SATA and sulfo-SMCC, both available from Pierce Chemical Co. (Rockford, IL).

**[0071]** Alternatively, the constituent molecules can be encoded in the same vector and expressed as a single protein in a host cell. Methods for producing such fusion proteins are described, for example, in U.S. Patent Number 5,260,203; U.S. Patent Number 5,455,030; U.S. Patent Number 4,881,175; U.S. Patent Number 5,132,405; U.S. Patent Number 5,091,513; U.S. Patent Number 5,476,786; U.S. Patent Number 5,013,653; U.S. Patent Number 5,258,498; and U.S. Patent Number 5,482,858.

**[0072]** In yet another embodiment, the invention provides Fn3-based binding molecules that are conjugated to poly-

ethylene glycol (PEG), for example, to increase the biological (e.g., serum) half-life of the molecule. Methods for PEGylating proteins are well known in the art. For example, the Fn3-based binding molecule can be reacted with a PEG moiety, such as a reactive ester or aldehyde derivative of PEG, under conditions in which one or more PEG groups become attached to the molecule. The term "PEGylation moiety", "polyethylene glycol moiety", or "PEG moiety" includes a polyalkylene glycol compound or a derivative thereof, with or without coupling agents or derivatization with coupling or activating moieties (e.g., with thiol, triflate, tresylate, aziridine, oxirane, or preferably with a maleimide moiety, e.g., PEG-maleimide). Other appropriate polyalkylene glycol compounds include, but are not limited to, maleimido monomethoxy PEG, activated PEG polypropylene glycol, but also charged or neutral polymers of the following types: dextran, colominic acids, or other carbohydrate based polymers, polymers of amino acids, and biotin derivatives.

**[0073]** The choice of the suitable functional group for the PEG derivative is based on the type of available reactive group on the molecule or molecule that will be coupled to the PEG. For proteins, typical reactive amino acids include lysine, cysteine, histidine, arginine, aspartic acid, glutamic acid, serine, threonine, tyrosine. The N-terminal amino group and the C-terminal carboxylic acid can also be used.

**[0074]** Preferably, the pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive PEG molecule (or an analogous reactive water-soluble polymer). As used herein, the term "polyethylene glycol" is intended to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono (C1-C10) alkoxy- or aryloxy-polyethylene glycol or polyethylene glycol-maleimide. Methods for pegylating proteins are known in the art and can be applied to the present invention. See for example, WO 2005056764, U.S.7,045,337, U.S.7,083,970, U.S. 6,927,042, EP 0 154 316 by Nishimura et al. and EP 0 401 384 by Ishikawa et al. Fn3-based binding molecules can be engineered to include at least one cysteine amino acid or at least one non-natural amino acid to facilitate pegylation.

**[0075]** Binding of the Fn3-based binding molecule conjugates to their specific targets can be confirmed by various assays, for example, the fusion can be radioactively labeled and used in a radioimmunoassay (RIA) (see, for example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986, which is incorporated by reference herein). The radioactive isotope can be detected by such means as the use of a  $\gamma$ - counter or a scintillation counter or by autoradiography.

**[0076]** Other Fn3-based binding molecule conjugates of the present invention include an Fn3-based binding molecule linked to a tag (e.g., biotin) or a chemical (e.g., an immunotoxin or chemotherapeutic agent). Such chemicals include cytotoxic agent which is any agent that is detrimental to (e.g., kills) cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents also include, for example, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine). Other examples of therapeutic cytotoxins that can be conjugated to Fn3-based binding molecule of the invention include duocarmycins, calicheamicins, maytansines and auristatins, and derivatives thereof.

**[0077]** Cytotoxins can be conjugated to the Fn3-based binding molecules of the invention using linker technology available in the art. Examples of linker types that have been used to conjugate a cytotoxin include, but are not limited to, hydrazones, thioethers, esters, disulfides and peptide-containing linkers. A linker can be chosen that is, for example, susceptible to cleavage by low pH within the lysosomal compartment or susceptible to cleavage by proteases, such as proteases preferentially expressed in tumor tissue such as cathepsins (e.g., cathepsins B, C, D). For further discussion of types of cytotoxins, linkers and methods for conjugating therapeutic agents, see also Saito, G. et al. (2003) Adv. Drug Deliv. Rev. 55:199-215; Trail, P.A. et al. (2003) Cancer Immunol. Immunother. 52:328-337; Payne, G. (2003) Cancer Cell 3:207-212; Allen, T.M. (2002) Nat. Rev. Cancer 2:750-763; Pastan, I. and Kreitman, R. J. (2002) Curr. Opin. Investig. Drugs 3:1089-1091; Senter, P.D. and Springer, C.J. (2001) Adv. Drug Deliv. Rev. 53:247-264.

**[0078]** Fn3-based binding molecules of the present invention also can be conjugated to a radioactive isotope to generate cytotoxic radiopharmaceuticals, also referred to as radioimmunoconjugates. Examples of radioactive isotopes that can be conjugated to Fn3-based binding molecules for use diagnostically or therapeutically include, but are not limited to, iodine<sup>131</sup>, indium<sup>111</sup>, yttrium<sup>90</sup> and lutetium<sup>177</sup>. Methods for preparing radioimmunoconjugates are established in the art. Examples of radioimmunoconjugates are commercially available, including ibritumomab, tiuxetan, and tositumomab, and similar methods can be used to prepare radioimmunoconjugates using the molecules of the invention.

**[0079]** The Fn3-based binding molecule conjugates of the invention can be used to modify a given biological response, and the drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, an enzymatically active toxin, or active fragment thereof, such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor or interferon- $\gamma$ ; or, biological response modifiers such as, for example,

lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

[0080] Techniques for conjugating such therapeutic moiety are well known and can be applied to the molecules of the present invention, see, e.g., Arnon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in *Monoclonal Antibodies And Cancer Therapy*, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al., "Antibodies For Drug Delivery", in *Controlled Drug Delivery (2nd Ed.)*, Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in *Monoclonal Antibodies '84: Biological And Clinical Applications*, Pinchera et al. (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in *Monoclonal Antibodies For Cancer Detection And Therapy*, Baldwin et al. (eds.), pp. 303-16 (Academic Press 1985), and Thorpe et al., "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", *Immunol. Rev.*, 62:119-58 (1982).

[0081] Fn3-based binding molecules of the present invention also can be modified by hesylation, which utilizes hydroxyethyl starch ("HES") derivatives linked to drug substances in order to modify the drug characteristics. HES is a modified natural polymer derived from waxy maize starch which is metabolized by the body's enzymes. This modification enables the prolongation of the circulation half-life by increasing the stability of the molecule, as well as by reducing renal clearance, resulting in an increased biological activity. Furthermore, HESylation potentially alters the immunogenicity or allergenicity. By varying different parameters, such as the molecular weight of HES, a wide range of HES drug conjugates can be customized.

[0082] DE 196 28 705 and DE 101 29 369 describe possible methods for carrying out the coupling of hydroxyethyl starch in anhydrous dimethyl sulfoxide (DMSO) via the corresponding aldono-lactone of hydroxyethyl starch with free amino groups of hemoglobin and amphotericin B, respectively. Since it is often not possible to use anhydrous, aprotic solvents specifically in the case of proteins, either for solubility reasons or else on the grounds of denaturation of the proteins, coupling methods with HES in an aqueous medium are also available. For example, coupling of hydroxyethyl starch which has been selectively oxidized at the reducing end of the chain to the aldonic acid is possible through the mediation of water-soluble carbodiimide EDC (1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide) (PCT/EP 02/02928). Additional hesylation methods which can be applied to the present invention are described, for example, in U.S. 20070134197, U.S. 20060258607, U.S. 20060217293, U.S. 20060100176, and U.S.20060052342.

[0083] Fn3-based binding molecules of the invention also can be modified via sugar residues. Methods for modifying sugar residues of proteins or glycosylating proteins are known in the art (see, for example, Borman (2006) *Chem. & Eng. News* 84(36):13-22 and Borman (2007) *Chem. & Eng. News* 85:19-20) and can be applied to the molecules of the present invention.

[0084] Additionally or alternatively, Fn3-based binding molecules of the invention can be made that have an altered type of glycosylation, such as a hypofucosylated pattern having reduced amounts of fucosyl residues or an Fn3-based binding molecule having increased bisecting GlcNAc structures. Such carbohydrate modifications can be accomplished by, for example, expressing the Fn3-based binding molecule in a host cell with altered glycosylation machinery. Cells with altered glycosylation machinery have been described in the art and can be used as host cells in which to express recombinant Fn3-based binding molecules of the invention to thereby produce Fn3-based binding molecules of the invention with altered glycosylation. For example, EP 1,176,195 by Hang et al. describes a cell line with a functionally disrupted FUT8 gene, which encodes a fucosyl transferase, such that antibodies expressed in such a cell line exhibit hypofucosylation. PCT Publication WO 03/035835 by Presta describes a variant CHO cell line, Lec13 cells, with reduced ability to attach fucose to Asn(297)-linked carbohydrates, also resulting in hypofucosylation of antibodies expressed in that host cell (see also Shields, R.L. et al., 2002 *J. Biol. Chem.* 277:26733-26740). Methods to produce polypeptides with human-like glycosylation patterns have also been described by EP1297172B1 and other patent families originating from Glycofi.

## Methods for Generating Fn3-Based Binding Molecules

### *1) Nucleic Acid and Amino Acid Alterations*

[0085] Fn3-based binding molecules of the invention having amino acid or nucleotide modifications (e.g., alterations) can be generated by a variety of known methods. Typically, such Fn3-based binding modified molecules are produced by recombinant methods. Moreover, because of the degeneracy of the genetic code, a variety of nucleic acid sequences can be used to encode each desired molecule.

[0086] Exemplary art recognized methods for making a nucleic acid molecule encoding an amino acid sequence variant of a starting molecule include, but are not limited to, preparation by site-directed (or oligonucleotide-mediated) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared DNA encoding the molecule.

[0087] Site-directed mutagenesis is a preferred method for preparing substitution variants. This technique is well known in the art (see, e.g., Carter et al. *Nucleic Acids Res.* 13:4431-4443 (1985) and Kunkel et al., *Proc. Natl. Acad.*

Sci. U.S.A 82:488 (1987)). Briefly, in carrying out site-directed mutagenesis of DNA, the parent DNA is altered by first hybridizing an oligonucleotide encoding the desired mutation to a single strand of such parent DNA. After hybridization, a DNA polymerase is used to synthesize an entire second strand, using the hybridized oligonucleotide as a primer, and using the single strand of the parent DNA as a template. Thus, the oligonucleotide encoding the desired mutation is incorporated in the resulting double-stranded DNA.

**[0088]** PCR mutagenesis is also suitable for making amino acid sequence variants of the starting molecule. See Higuchi, in PCR Protocols, pp. 177-183 (Academic Press, 1990); and Vallette et al., Nuc. Acids Res. 17:723-733 (1989). Briefly, when small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template.

**[0089]** Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells et al., Gene 34:315-323 (1985). The starting material is the plasmid (or other vector) comprising the starting polypeptide DNA to be mutated. The codon(s) in the parent DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the starting polypeptide DNA. The plasmid DNA is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures, wherein the two strands of the oligonucleotide are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 5' and 3' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated DNA sequence.

**[0090]** Alternatively, or additionally, the desired amino acid sequence encoding a polypeptide variant of the molecule can be determined, and a nucleic acid sequence encoding such amino acid sequence variant can be generated synthetically.

**[0091]** It will be understood by one of ordinary skill in the art that the Fn3-based binding molecules of the invention may further be modified such that they vary in amino acid sequence (e.g., from wild-type), but not in desired activity. For example, additional nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues may be made to the protein. For example, a nonessential amino acid residue in a molecule may be replaced with another amino acid residue from the same side chain family. In another embodiment, a string of amino acids can be replaced with a structurally similar string that differs in order and/or composition of side chain family members, i.e., a conservative substitution, in which an amino acid residue is replaced with an amino acid residue having a similar side chain, may be made.

**[0092]** Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

**[0093]** Aside from amino acid substitutions, the present invention contemplates other modifications of the starting molecule amino acid sequence in order to generate functionally equivalent molecules. For example, one may delete one or more amino acid residues. Generally, no more than one to about ten residues will be deleted according to this embodiment of the invention. The Fn3-based binding molecules comprising one or more amino acid deletions will preferably retain at least about 80%, and preferably at least about 90%, and most preferably at least about 95%, of the starting polypeptide molecule.

**[0094]** One may also make amino acid insertion variants, which retain the original Fn3 domain or Fn3-based binding molecule functionality. For example, one may introduce at least one amino acid residue (e.g. one to two amino acid residues and generally no more than ten residues) into the molecule. In another embodiment amino acid modifications may be combined within an Fn3 domain or single Fn3-based binding molecules.

**[0095]** In one embodiment, amino acid substitutions are performed on an Fn3 domain to include cysteine or other non-natural amino acid suitable for conjugating a moiety to the Fn3-based binding molecule using well-known conjugating methods. In particular, the invention relates to specific amino acid variants of Fn3-based binding molecule with Fn3 scaffold, wherein one or more serine amino acid residues are substituted by cysteine or a non-natural amino acid. Serine amino acid residues that can be substituted include, but are not limited to Ser 1432, Ser 1436, Ser 1458, Ser 1475, and Ser 1504. Other amino acid positions of the Fn3 scaffold that can be substituted include, but are not limited to, V1426, L1434, T1473 and T1486. Non-naturally occurring amino acids can be substituted into the Fn3 scaffold using, for example, Ambrex technology (See e.g., US 7,045,337; 7,083,970).

## 2) Screening Assays for Identifying Fn3-Based Binding Molecules

**[0096]** A variety of screening assays can be employed to identify Fn3-based binding molecules of the invention. Essentially any *in vitro* or *in vivo* screening method that selects for binding to a desired antigen can be used.

**[0097]** In one embodiment, Fn3-based binding molecules are displayed on the surface of a cell, virus or bacteriophage and subject to selection using immobilized antigen. Suitable methods of screening are described in U.S. patent numbers 7,063,943; 6,699,658; 7,063,943 and 5866344. Such surface display may require the creation of fusion proteins of the Fn3-based binding molecules with a suitable protein normally present on the outer surface of a cell, virus or bacteriophage. Suitable proteins from which to make such fusions are well known in the art.

**[0098]** In another embodiment, Fn3-based binding molecules are screened using an *in vitro* phenotype-genotype linked display such as ribosome or polysome display. Such methods of "molecular evolution" are well known in the art (see for example U.S. patent number 6,194,550 and 7,195,880).

**[0099]** Screening methods may involve one or more *in vitro* or *in vivo* affinity maturation steps. Any affinity maturation approach can be employed that results in amino acid changes in the Fn3 domain or the CDRs that improve the binding of the Fn3-based binding molecule to the desired antigen. These amino acid changes can, for example, be achieved via random mutagenesis, "walk through mutagenesis, and "look through mutagenesis. Such mutagenesis can be achieved by using, for example, error-prone PCR, "mutator" strains of yeast or bacteria, incorporation of random or defined nucleic acid changes during *ab initio* synthesis of all or part of a Fn3-based binding molecule. Methods for performing affinity maturation and/or mutagenesis are described, for example, in U.S. Patent Numbers 7,195,880; 6,951,725; 7,078,197; 7,022,479; 5,922,545; 5,830,721; 5,605,793, 5,830,650; 6,194,550; 6,699,658; 7,063,943; 5866344 and Patent Cooperation Treaty publication WO06023144. Such affinity maturation methods may further require that the stringency of the antigen-binding screening assay is increased to select for Fn3-based binding molecules with improved affinity for antigen. Art recognized methods for increasing the stringency of a protein-protein interaction assay can be used here. In one embodiment, one or more of the assay conditions are varied (for example, the salt concentration of the assay buffer) to reduce the affinity of the Fn3-based binding molecules for the desired antigen. In another embodiment, the length of time permitted for the Fn3-based binding molecules to bind to the desired antigen is reduced. In another embodiment, a competitive binding step is added to the protein-protein interaction assay. For example, the Fn3-based binding molecules are first allowed to bind to a desired immobilized antigen. A specific concentration of non-immobilized antigen is then added which serves to compete for binding with the immobilized antigen such that the Fn3-based binding molecules with the lowest affinity for antigen are eluted from the immobilized antigen resulting in selection of Fn3-based binding molecules with improved antigen binding affinity. The stringency of the assay conditions can be further increased by increasing the concentration of non-immobilized antigen is added to the assay.

**[0100]** Screening methods of the invention may also require multiple rounds of selection to enrich for one or more Fn3-based binding molecules with improved antigen binding. In one embodiment, at each round of selection further amino acid mutation are introduced into the Fn3-based binding molecules. In another embodiment, at each round of selection the stringency of binding to the desired antigen is increased to select for Fn3-based binding molecules with increased affinity for antigen.

**[0101]** In the case of the bispecific Fn3-based binding molecules of the invention, it is preferable to screen for each binding specificity independently. Accordingly, a first screen to identify individual Fn3-based binding molecules, that bind to a first target, is performed using a first library of Fn3-based binding molecules, where one or more amino acids in one or more of the AB, CD or EF loops is altered. A second separate screen to identify individual Fn3-based binding molecules, that bind to a second target, is performed using a second library of Fn3 domain molecules, where one or more amino acids in one or more of the BC, DE and FG loops is altered. The amino acid sequences of the individual monospecific Fn3-based binding molecules identified from both screens are determined using art recognized methods. Bispecific Fn3-based binding molecules are generated by combining first and second target-binding sequences from individual Fn3-based binding molecules into single, chimeric Fn3-based binding molecules.

## 3) Methods of Manufacture

**[0102]** The Fn3-based binding molecules of the invention are typically produced by recombinant expression. Nucleic acids encoding the molecules are inserted into expression vectors. The DNA segments encoding the molecules are operably linked to control sequences in the expression vector(s) that ensure their expression. Expression control sequences include, but are not limited to, promoters (e.g., naturally-associated or heterologous promoters), signal sequences, enhancer elements, and transcription termination sequences. Preferably, the expression control sequences are eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences, and the collection and purification of the crossreacting Fn3-based binding molecule.

**[0103]** These expression vectors are typically replicable in the host organisms either as episomes or as an integral

part of the host chromosomal DNA. Commonly, expression vectors contain selection markers (e.g., ampicillin-resistance, hygromycin-resistance, tetracycline resistance or neomycin resistance) to permit detection of those cells transformed with the desired DNA sequences (see, e.g., Itakura et al., U.S. Patent 4,704,362).

5 [0104] *E. coli* is one prokaryotic host particularly useful for cloning the polynucleotides (e.g., DNA sequences) of the present invention. Other microbial hosts suitable for use include bacilli, such as *Bacillus subtilis*, and other enterobacteriaceae, such as *Salmonella*, *Serratia*, and various *Pseudomonas* species.

[0105] Other microbes, such as yeast, are also useful for expression. *Saccharomyces* and *Pichia* are exemplary yeast hosts, with suitable vectors having expression control sequences (e.g., promoters), an origin of replication, termination sequences and the like as desired. Typical promoters include 3-phosphoglycerate kinase and other glycolytic enzymes. 10 Inducible yeast promoters include, among others, promoters from alcohol dehydrogenase, isocytochrome C, and enzymes responsible for methanol, maltose, and galactose utilization.

[0106] In addition to microorganisms, mammalian tissue culture may also be used to express and produce the polypeptides of the present invention (e.g., polynucleotides encoding immunoglobulins or fragments thereof). See Winnacker, From Genes to Clones, VCH Publishers, N.Y., N.Y. (1987). Eukaryotic cells are actually preferred, because a number 15 of suitable host cell lines capable of secreting heterologous proteins (e.g., intact immunoglobulins) have been developed in the art, and include CHO cell lines, various COS cell lines, HeLa cells, 293 cells, myeloma cell lines, transformed B-cells, and hybridomas. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, and an enhancer (Queen et al., Immunol. Rev. 89:49 (1986)), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, SV40, adenovirus, bovine papilloma virus, cytomegalovirus and the like. See Co et al., J. Immunol. 148:1149 (1992).

[0107] Alternatively, coding sequences can be incorporated in transgenes for introduction into the genome of a transgenic animal and subsequent expression in the milk of the transgenic animal (see, e.g., Deboer et al., U.S. 5,741,957, 20 Rosen, U.S. 5,304,489, and Meade et al., U.S. 5,849,992). Suitable transgenes include coding sequences for light and/or heavy chains in operable linkage with a promoter and enhancer from a mammary gland specific gene, such as casein or beta lactoglobulin.

[0108] The vectors containing the polynucleotide sequences of interest and expression control sequences can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, 25 chemically competent prokaryotic cells may be briefly heat-shocked, whereas calcium phosphate treatment, electroporation, lipofection, biolistics or viral-based transfection may be used for other cellular hosts. (See generally Sambrook et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Press, 2nd ed., 1989). Other methods used to transform mammalian cells include the use of polybrene, protoplast fusion, liposomes, electroporation, and microinjection (see generally, Sambrook et al., supra). For production of transgenic animals, transgenes can be microinjected into fertilized oocytes, or can be incorporated into the genome of embryonic stem cells, and the nuclei of such cells transferred 30 into enucleated oocytes.

[0109] Once expressed, the Fn3-based binding molecules of the present invention can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, HPLC purification, gel electrophoresis and the like (see generally Scopes, Protein Purification (Springer-Verlag, N.Y., (1982)). Substantially pure molecules of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity 35 most preferred, for pharmaceutical uses.

#### 4) Methods for Grafting CDRs onto Fn3-Based Binding Molecules

[0110] In one aspect, the present invention features an Fn3-based binding molecule altered compared to the wild-type Fn3 domain to contain all or a portion of a complementarity determining region (CDR) of an antibody or a T-cell 40 receptor.

[0111] The CDR regions of any antibody or T-cell receptor variable region, or antigen binding fragments thereof, are suitable for grafting. The CDRs can be obtained from the antibody or T-cell receptor repertoire of any animal including, but not limited to, rodents, primates, camelids or sharks. In a particular embodiment, the CDRs are obtained from CDR1, 45 CDR2 and CDR3 of a single domain antibody, for example a nanobody. In a more specific embodiment, CDR1, 2 or 3 of a single domain antibody, such as a nanobody, are grafted into any of the AB, BC, CD, DE, EF or FG loops of an Fn3 domain, thereby providing target binding specificity of the original nanobody to the Fn3-based binding molecule. Engineered libraries of camelid antibodies and antibody fragments are commercially available, for example, from Ablynx, Ghent, Belgium. The antibody repertoire can be from animals challenged with one or more antigens or from naive animals 50 that have not been challenged with antigen. Additionally or alternatively, CDRs can be obtained from antibodies, or antigen binding fragments thereof, produced by *in vitro* or *in vivo* library screening methods, including, but not limited to, *in vitro* polysome or ribosome display, phage display or yeast display techniques. This includes antibodies not originally generated by *in vitro* or *in vivo* library screening methods but which have subsequently undergone mutagenesis or one 55

or more affinity maturation steps using *in vitro* or *in vivo* screening methods. Example of such *in vitro* or *in vivo* library screening methods or affinity maturation methods are described, for example, in U.S. Patent Numbers 7,195,880; 6,951,725; 7,078,197; 7,022,479; 5,922,545; 5,830,721; 5,605,793, 5,830,650; 6,194,550; 6,699,658; 7,063,943; 5866344 and Patent Cooperation Treaty publications WO06023144.

**[0112]** Methods to identify antibody CDRs are well known in the art (see Kabat et al., U.S. Dept. of Health and Human Services, "Sequences of Proteins of Immunological Interest" (1983); Chothia et al., J. Mol. Biol. 196:901-917 (1987); MacCallum et al., J. Mol. Biol. 262:732-745 (1996)). The nucleic acid encoding a particular antibody can be isolated and sequenced, and the CDR sequences deduced by inspection of the encoded protein with regard to the established antibody sequence nomenclature. Methods for grafting hypervariable regions or CDRs into a Fn3-based binding scaffold of the invention include, for example, genetic engineering, *de novo* nucleic acid synthesis or PCR-based gene assembly (see for example U.S. patent number 5,225,539).

**[0113]** The above techniques allow for the identification of a suitable scaffold loop for selection and presentation of a hypervariable region or CDR. However, additional metrics can be invoked to further improve the fit and presentation of the hypervariable region based on structural modeling of the Fn3 domain and the donor antibody.

**[0114]** In one aspect, specific amino acid residues in any of the beta-strands of an Fn3 domain are mutated to allow the CDR loops to adopt a conformation that retains or improves binding to antigen. This procedure can be performed in an analogous way to that CDR grafting into a heterologous antibody framework, using a combination of structural modeling and sequence comparison. In one embodiment, the Fn3 domain residues adjacent to a CDR are mutated in a similar manner to that performed by Queen *et al.* (see U.S. patent numbers 6,180,370; 5,693,762; 5,693,761; 5,585,089; 7,022,500). In another embodiment, Fn3 domain residues within one Van der Waals radius of CDR residues are mutated in a similar manner to that performed by Winter *et al.* (see U.S. patent numbers 6,548,640; 6,982,321). In another embodiment, Fn3 domain residues that are non-adjacent to CDR residues but are predicted, based upon structural modeling of the Fn3 domain and the donor antibody, to modify the conformation of CDR residues are mutated in a similar manner to that performed by Carter *et al.* or Adair *et al.* (see U.S. patent numbers 6,407,213; 6,639,055; 5,859,205; 6,632,927).

#### Compositions

**[0115]** The Fn3-based binding of the present invention have *in vivo* therapeutic utilities. Accordingly, the present invention also provides compositions, *e.g.*, a pharmaceutical composition, containing one or a combination of Fn3-based binding molecules (or variants, fusions, and conjugates thereof), formulated together with a pharmaceutically acceptable carrier. Pharmaceutical compositions of the invention also can be administered in combination therapy, *i.e.*, combined with other agents. For example, the combination therapy can include a composition of the present invention with at least one or more additional therapeutic agents, such as anti-inflammatory agents, anti-cancer agents, and chemotherapeutic agents.

**[0116]** The pharmaceutical compositions of the invention can also be administered in conjunction with radiation therapy. Co-administration with other Fn3-based molecules are also encompassed by the invention.

**[0117]** As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Preferably, the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (*e.g.*, by injection or infusion). Depending on the route of administration, the active compound, *i.e.*, antibody, bispecific and multispecific molecule, may be coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound.

**[0118]** A "pharmaceutically acceptable salt" refers to a salt that retains the desired biological activity of the parent compound and does not impart any undesired toxicological effects (see *e.g.*, Berge, S.M., et al. (1977) J. Pharm. Sci. 66:1-19). Examples of such salts include acid addition salts and base addition salts. Acid addition salts include those derived from nontoxic inorganic acids, such as hydrochloric, nitric, phosphoric, sulfuric, hydrobromic, hydroiodic, phosphorous and the like, as well as from nontoxic organic acids such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanic acids, hydroxy alkanic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts include those derived from alkaline earth metals, such as sodium, potassium, magnesium, calcium and the like, as well as from nontoxic organic amines, such as N,N'-dibenzylethylenediamine, N-methylglucamine, chlorprocaine, choline, diethanolamine, ethylenediamine, procaine and the like.

**[0119]** A composition of the present invention can be administered by a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. The active compounds can be prepared with carriers that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or

generally known to those skilled in the art. See, e.g., Sustained and Controlled Release Drug Delivery Systems, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

5 [0120] To administer a compound of the invention by certain routes of administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. For example, the compound may be administered to a subject in an appropriate carrier, for example, liposomes, or a diluent. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes (Strejan et al. (1984) J. Neuroimmunol. 7:27).

10 [0121] Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

15 [0122] Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

20 [0123] Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by sterilization microfiltration. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying (lyophilization) that yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

25 [0124] Dosage regimens are adjusted to provide the optimum desired response (e.g., a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. For example, the Fn3-based binding molecule of the invention may be administered once or twice weekly by subcutaneous injection or once or twice monthly by subcutaneous injection. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

30 [0125] Examples of pharmaceutically-acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

35 [0126] For the therapeutic compositions, formulations of the present invention include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the subject being treated, and the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the composition which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 0.001 per cent to about ninety percent of active ingredient, preferably from about 0.005 per cent to about 70 per cent, most preferably from about 0.01 per cent to about 30 per cent.

40 [0127] Formulations of the present invention which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate. Dosage forms for the topical or transdermal administration of compositions of this invention include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants

which may be required.

**[0128]** The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

**[0129]** Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

**[0130]** These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of microorganisms may be ensured both by sterilization procedures, supra, and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

**[0131]** When the compounds of the present invention are administered as pharmaceuticals, to humans and animals, they can be given alone or as a pharmaceutical composition containing, for example, 0.001 to 90% (more preferably, 0.005 to 70%, such as 0.01 to 30%) of active ingredient in combination with a pharmaceutically acceptable carrier.

**[0132]** Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art.

**[0133]** Actual dosage levels of the active ingredients in the pharmaceutical compositions of the present invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts. A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. In general, a suitable daily dose of a compositions of the invention will be that amount of the compound which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. It is preferred that administration be intravenous, intramuscular, intraperitoneal, or subcutaneous, preferably administered proximal to the site of the target. If desired, the effective daily dose of therapeutic compositions may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms. While it is possible for a compound of the present invention to be administered alone, it is preferable to administer the compound as a pharmaceutical formulation (composition).

**[0134]** Therapeutic compositions can be administered with medical devices known in the art. For example, in a preferred embodiment, a therapeutic composition of the invention can be administered with a needleless hypodermic injection device, such as the devices disclosed in U.S. Patent Nos. 5,399,163, 5,383,851, 5,312,335, 5,064,413, 4,941,880, 4,790,824, or 4,596,556. Examples of well-known implants and modules useful in the present invention include: U.S. Patent No. 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; U.S. Patent No. 4,486,194, which discloses a therapeutic device for administering medicants through the skin; U.S. Patent No. 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion rate; U.S. Patent No. 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; U.S. Patent No. 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments; and U.S. Patent No. 4,475,196, which discloses an osmotic drug delivery system. Many other such implants, delivery systems, and modules are known to those skilled in the art.

**[0135]** In certain embodiments, the molecules of the invention can be formulated to ensure proper distribution *in vivo*. For example, the blood-brain barrier (BBB) excludes many highly hydrophilic compounds. To ensure that the therapeutic compounds of the invention cross the BBB (if desired), they can be formulated, for example, in liposomes. For methods of manufacturing liposomes, see, e.g., U.S. Patents 4,522,811; 5,374,548; and 5,399,331. The liposomes may comprise one or more moieties which are selectively transported into specific cells or organs, thus enhance targeted drug delivery

(see, e.g., V.V. Ranade (1989) J. Clin. Pharmacol. 29:685). Exemplary targeting moieties include folate or biotin (see, e.g., U.S. Patent 5,416,016 to Low et al.); mannosides (Umezawa et al., (1988) Biochem. Biophys. Res. Commun. 153:1038); antibodies (P.G. Bloeman et al. (1995) FEBS Lett. 357:140; M. Owais et al. (1995) Antimicrob. Agents Chemother. 39:180); surfactant protein A receptor (Briscoe et al. (1995) Am. J. Physiol. 1233:134), different species of which may comprise the formulations of the inventions, as well as components of the invented molecules; p120 (Schreier et al. (1994) J. Biol. Chem. 269:9090); see also K. Keinanen; M.L. Laukkanen (1994) FEBS Lett. 346:123; J.J. Killion; I.J. Fidler (1994) Immunomethods 4:273. In one embodiment of the invention, the therapeutic compounds of the invention are formulated in liposomes; in a more preferred embodiment, the liposomes include a targeting moiety. In a most preferred embodiment, the therapeutic compounds in the liposomes are delivered by bolus injection to a site proximal to the tumor or infection. The composition must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi.

**[0136]** In a further embodiment, the molecules of the invention can be formulated to prevent or reduce the transport across the placenta. This can be done by methods known in the art, e.g., by PEGylation of the Fn3-based binding molecule. Further references can be made to "Cunningham-Rundles C, Zhuo Z, Griffith B, Keenan J. (1992) Biological activities of polyethylene-glycol immunoglobulin conjugates. Resistance to enzymatic degradation. J Immunol Methods. 152:177-190; and to "Landor M. (1995) Maternal-fetal transfer of immunoglobulins, Ann Allergy Asthma Immunol 74: 279-283. This is particularly relevant when the Fn3-based binding molecules are used for treating or preventing recurrent spontaneous abortion.

**[0137]** The ability of a compound to inhibit cancer can be evaluated in an animal model system predictive of efficacy in human tumors. Alternatively, this property of a composition can be evaluated by examining the ability of the compound to inhibit, such inhibition *in vitro* by assays known to the skilled practitioner. A therapeutically effective amount of a therapeutic compound can decrease tumor size, or otherwise ameliorate symptoms in a subject. One of ordinary skill in the art would be able to determine such amounts based on such factors as the subject's size, the severity of the subject's symptoms, and the particular composition or route of administration selected.

**[0138]** The composition must be sterile and fluid to the extent that the composition is deliverable by syringe. In addition to water, the carrier can be an isotonic buffered saline solution, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. Proper fluidity can be maintained, for example, by use of coating such as lecithin, by maintenance of required particle size in the case of dispersion and by use of surfactants. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol or sorbitol, and sodium chloride in the composition. Long-term absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate or gelatin.

**[0139]** When the active compound is suitably protected, as described above, the compound may be orally administered, for example, with an inert diluent or an assimilable edible carrier.

#### Therapeutic and Diagnostic Applications

**[0140]** The Fn3-based binding molecules described herein may be constructed to bind any antigen or target of interest. Such targets include, but are not limited to, cluster domains, cell receptors, cell receptor ligands, growth factors, interleukins, protein allergens, bacteria, or viruses (see, Figure 1). The Fn3-based binding molecules described herein may also be modified to have increased stability and half-life, as well as additional functional moieties. Accordingly, these molecules may be employed in place of antibodies in all areas in which antibodies are used, including in the research, therapeutic, and diagnostic fields. In addition, because these molecules possess solubility and stability properties superior to antibodies, the antibody mimics described herein may also be used under conditions which would destroy or inactivate antibody molecules.

**[0141]** For example, these molecules can be administered to cells in culture, e.g. *in vitro* or *ex vivo*, or in a subject, e.g., *in vivo*, to treat, prevent or diagnose a variety of disorders. The term "subject" as used herein intended to include human and non-human animals. Non-human animals includes all vertebrates, e.g., mammals and non-mammals, such as non-human primates, sheep, dogs, cats, cows, horses, chickens, amphibians, and reptiles. When the Fn3 molecules are administered together with another agent, the two can be administered in either order or simultaneously.

**[0142]** In one embodiment, the Fn3-based binding molecules (and variants, fusions, and conjugates thereof) of the invention can be used to detect levels of the target bound by the molecule and/or the targets bound by a bispecific/multispecific Fn3-based binding molecule. This can be achieved, for example, by contacting a sample (such as an *in vitro* sample) and a control sample with the molecule under conditions that allow for the formation of a complex between the molecule and the target(s). Any complexes formed between the molecule and the target(s) are detected and compared in the sample and the control. For example, standard detection methods, well-known in the art, such as ELISA, FACS, and flow cytometric assays, can be performed using the compositions of the invention.

**[0143]** Also within the scope of the disclosure are kits comprising the compositions (e.g., Fn3-based binding molecules,

variants, fusions, and conjugates thereof) of the invention and instructions for use. The kit can further contain a least one additional reagent, or one or more additional Fn3-based binding molecules of the invention (e.g., an antibody having a complementary activity which binds to an epitope on the target antigen distinct from the first molecule). Kits typically include a label indicating the intended use of the contents of the kit. The term label includes any writing, or recorded material supplied on or with the kit, or which otherwise accompanies the kit.

**[0144]** As described above, the molecules of the present invention may be employed in all areas of the research, therapeutic, and diagnostic fields. Exemplary diseases/disorders which can be treated using the Fn3-based binding molecules of the present invention (and variants, fusions, and conjugates thereof) include autoimmune disorders, cancers, infections, and other pathogenic indications.

**[0145]** Specific examples of autoimmune conditions in which the Fn3-based binding molecules of the invention can be used include, but are not limited to, the following: multiple sclerosis and other demyelinating diseases; rheumatoid arthritis; inflammatory bowel disease; systemic lupus erythematosus; Type I diabetes; inflammatory skin disorders; Sjogren's Syndrome; and transplant rejection.

**[0146]** Specific examples of cancers in which the Fn3-based binding molecules of the invention can be used include, but are not limited to, the following: lung; breast; prostate; bladder; melanoma; non-Hodgkin lymphoma; colon and rectal; pancreatic; endometrial; kidney; skin (non-melanoma); leukemia; and thyroid.

**[0147]** Specific examples of diseases associated with VEGF include for example, a number of conditions associated with inappropriate angiogenesis, including but not limited to autoimmune disorders (e.g., rheumatoid arthritis, inflammatory bowel disease or psoriasis); cardiac disorders (e.g., atherosclerosis or blood vessel restenosis); retinopathies (e.g., proliferative retinopathies generally, diabetic retinopathy, age-related macular degeneration or neovascular glaucoma), renal disease (e.g., diabetic nephropathy, malignant nephrosclerosis, thrombotic microangiopathy syndromes; transplant rejection; inflammatory renal disease; glomerulonephritis; mesangioproliferative glomerulonephritis; haemolytic-uraemic syndrome; and hypertensive nephrosclerosis); hemangioblastoma; hemangiomas; thyroid hyperplasias; tissue transplantations; chronic inflammation; Meigs's syndrome; pericardial effusion; pleural effusion; autoimmune diseases; diabetes; endometriosis; chronic asthma; undesirable fibrosis (particularly hepatic fibrosis) and cancer, as well as complications arising from cancer, such as pleural effusion and ascites. The Fn3-based binding molecules can be used for the treatment of prevention of hyperproliferative diseases or cancer and the metastatic spread of cancers. Non-limiting examples of cancers include bladder, blood, bone, brain, breast, cartilage, colon kidney, liver, lung, lymph node, nervous tissue, ovary, pancreatic, prostate, skeletal muscle, skin, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, urogenital tract, ureter, urethra, uterus, or vaginal cancer. Additional treatable conditions can be found in U.S.P.N. 6,524, 583, herein incorporated by reference. Other references describing uses for VEGFR-2 binding polypeptides include: McLeod DS et al., Invest Ophthalmol Vis Sci. 2002 Feb;43(2):474-82; Watanabe et al. Exp Dermatol. 2004 Nov;13(11): 671-81; Yoshiji H et al., Gut. 2003 Sep;52(9): 1347-54; Verheul et al., Oncologist. 2000;5 Suppl 1:45-50; Boldicke et al., Stem Cells. 2001;19(1):24-36. As described herein, angiogenesis-associated diseases include, but are not limited to, angiogenesis-dependent cancer, including, for example, solid tumors, blood born tumors such as leukemias, and tumor metastases; benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; inflammatory disorders such as immune and non-immune inflammation; chronic articular rheumatism and psoriasis; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophilic joints; angiofibroma; and wound granulation and wound healing; telangiectasia psoriasis scleroderma, pyogenic granuloma, coronary collaterals, ischemic limb angiogenesis, corneal diseases, rubeosis, arthritis, diabetic neovascularization, fractures, vasculogenesis, hematopoiesis (see e.g., W02005056764).

**[0148]** Specific examples of infections in which the Fn3-based binding molecules of the invention can be used include, but are not limited to, the following: cellular, fungal, bacterial, and viral.

**[0149]** The present invention is further illustrated by the following examples which should not be construed as further limiting. The contents of all figures and all references, patents and published patent applications cited throughout this application are expressly incorporated herein by reference.

## **Exemplification**

### **Example 1**

#### Production of Libraries of Fibronectin-Based Binding Molecules

**[0150]** In general, the practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, recombinant DNA technology, immunology (especially, e.g., antibody technology), and standard techniques in polypeptide preparation. See, e.g., Sambrook, Fritsch and Maniatis, Molecular Cloning: Cold

## EP 2 274 331 B1

Spring Harbor Laboratory Press (1989); Antibody Engineering Protocols (Methods in Molecular Biology), 510, Paul, S., Humana Pr (1996); Antibody Engineering: A Practical Approach (Practical Approach Series, 169), McCafferty, Ed., Irl Pr (1996); Antibodies: A Laboratory Manual, Harlow et al., C.S.H.L. Press, Pub. (1999); and Current Protocols in Molecular Biology, eds. Ausubel et al., John Wiley & Sons (1992). Other methods, techniques, and sequences suitable for use in carrying out the present invention are found in U.S. Pat. Nos. 7,153,661; 7,119,171; 7,078,490; 6,703,199; 6,673,901; and 6,462,189.

**[0151]** The 10th human fibronectin protein (Fn<sup>3</sup><sub>10</sub>) was used to design, isolate, and engineer monospecific binders. Initial binders were first independently isolated from two libraries using standard selection methods. This enriched population was then mutagenized, and successive rounds of random mutagenesis and enrichment were conducted to attain desired monospecific binders.

### *Library Construction*

**[0152]** Wildtype Fn<sup>3</sup><sub>10</sub> sequence as shown in SEQ ID NO: 1 was used as the basis to generate libraries of binders that utilize the top or bottom loops.

VSDVPRDLEVVAATPTSLISWDAPAVTVRYRITYGETGGNSPVQEFTVPGSK  
STATISGLKPGVDYITITVYAVTGRGDSPASSKPISINYRTEI (SEQ ID NO: 1)

**[0153]** Using computational modeling, two sets of variable regions of the wildtype Fn<sup>3</sup><sub>10</sub> were chosen to be randomized. The first set comprising the solvent exposed top loops BC, DE and FG was designated library A and was randomized in the region shown in boldface in SEQ ID NO: 2.

**[0154]** Library A (beta-sandwich with solvent exposed top loops BC, DE and FG).

VSDVPRDLEVVAATPTSLISWDAPAVTVRYRITYGETGGNSPVQEFTVPGSK  
STATISGLKPGVDYITITVYAVTGRGDSPASSKPISINYRTEI (SEQ ID NO: 2)

**[0155]** The second set comprising the solvent exposed bottom loops AB, CD, EF and C-terminus was designated library B and was randomized in the underlined regions shown in italics.

**[0156]** Library B (beta-sandwich with solvent exposed bottom loops AB, CD, EF and C-terminus).

VSDVPRDLEVVAATPTSLISWDAPAVTVRYRITYGETGGNSPVQEFTVPGSKS  
TATISGLKPGVDYITITVYAVTGRGDSPASSKPISINYRTEI (SEQ ID NO: 3)

**[0157]** The DNA sequences corresponding to the two libraries were optimised for expression in *E.coli* at Genent AG, Germany. Regions in boldface or underlined italics from SEQ ID NOs: 2 and 3, respectively, were synthesized as degenerated positions. The libraries were assembled from synthetic degenerated oligonucleotides and genes corresponding to full length fragments gel purified. Amplification was performed with terminal primers and subsequent ligation of the amplified library into cloning vector pCR-Script yielded the starting libraries. Libraries A and B were then independently screened to identify monospecific binders as described in Example 2 below.

### **Example 2**

#### Screening of Monospecific Fibronectin-Based Binding Molecules

**[0158]** The present Example describes how to screen for fibronectin monospecific binders generated from the Libraries A and B described in Example 1. Both libraries A and B were independently subcloned into a yeast display vector such as pYD1 (Invitrogen) using homologous recombination methods and transformed into a suitable strain such as EBY100 using standard molecular biology techniques.

**[0159]** Presentation and selection of fibronectin based binders against hen egg lysozyme was conducted following essentially the protocol previously published by Lipovsek, D. et al, (J Mol Biol. 2007 May 11;368(4):1024-41) with some minor modifications. Both libraries were independently screened for binders to hen egg white lysozyme.

## EP 2 274 331 B1

### (i) Selection for binding to hen egg white lysozyme using magnetic bead sorting

**[0160]** For all selections, yeast cultures presenting library A or library B of <sup>10</sup>F<sub>n</sub>3-based molecules were induced for 18 h at 30°C in galactose-containing medium (90% SG-CAA/10% SD-CAA, 50 µg/mL kanamycin, 100 U/mL penicillin G, 200 U/mL streptomycin). 10<sup>9</sup> induced yeast cells of libraries A or B were washed with 25 mL of ice-cold phosphate-buffered saline (PBS), pH 7.4, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.5% bovine serum albumin (BSA) and then incubated in 5 mL of the same buffer containing 1 µM biotinylated hen egg white lysozyme (HEL-b, Sigma, St. Louis, MO) for 1 h at room temperature with gentle rotation. Following the incubation, the sample was chilled on ice, washed with 25 mL of ice-cold PBS, pH 7.4, 2 mM EDTA, 0.5% BSA and resuspended in 2.5 mL of the same buffer. 100-µL aliquot of magnetic Streptavidin MicroBeads (Miltenyi Biotec, Auburn, CA) was added to the yeast and incubated on ice for 10 min. Ice-cold PBS, pH 7.4, 2 mM EDTA, 0.5% BSA was added to the sample to a total volume of 25 mL immediately before it was subjected to separation on an AutoMACS Cell Separator (Miltenyi Biotec), using the preset program for positive selection of rare cells (possel\_s). Selected cells were collected in 6 mL SD-CAA, pH 4.5, 50 µg/mL kanamycin, 100 U/mL penicillin G, 200 U/mL streptomycin; quantified by serial dilution followed by plating on SD-CAA agar plates; and grown in 50 mL of the same medium for 2 days at 30°C.

### (ii) Selection for binding to hen egg white lysozyme using fluorescence-activated cell sorting

**[0161]** Subsequent rounds of selection were performed by FACS, starting with 2 × 10<sup>6</sup> to 3 × 10<sup>6</sup> induced yeast cells. Cells were washed with 1 mL PBS, pH 7.4, 0.1% BSA, resuspended in 100 µL of the same buffer containing biotinylated hen egg white lysozyme, and incubated at room temperature with gentle rotation for 1 h.

**[0162]** After being washed with 1 mL of ice-cold PBS, pH 7.4, 0.1% BSA, the cells were labeled with antibodies and streptavidin. Mouse monoclonal FITC-conjugated anti-c-myc antibody (AbD Serotec) was used to label the yeast for surface display of c-myc-tagged antibody mimics, and PE-labeled streptavidin (Invitrogen) or anti-biotin antibody (Miltenyi) was used to label HEL-b associated with lysozyme-binding antibody mimics. The FACS sorts were performed on yeast cells labeled with FITC-conjugated mouse anti-c-myc antibody and PE-conjugated streptavidin (Invitrogen).

**[0163]** Double-labeled yeast cells were sorted on a Dako MoFlo high-speed cell sorter with a 488 nm laser, at 6000-10,000 cells/s. Gates were adjusted to collect the yeast cells with the highest 0.1-1% of HEL-b-associated signal (PE) and in the top half of expression-associated signal (FITC). Duplicate samples labeled with the same antibody and streptavidin reagents, but in the absence of HEL-b were used to avoid selecting the cells that bound detection reagents instead of lysozyme.

**[0164]** For all libraries, the first two FACS sorts were performed on yeast labeled with 1 µM HEL-b. Once a population of cells was observed that was labeled with PE in the presence but not in the absence of HEL-b, the concentration of HEL-b in the subsequent round was decreased by an order of magnitude. Selected cells were collected in 0.5 mL of SD-CAA, pH 4.5, 50 µg/mL kanamycin, 100 U/mL penicillin G, and 200 U/mL streptomycin. The collected cells were grown to saturation in 5 mL of the same medium, with shaking, for 2 days at 30 °C, before being induced and labeled for the next round of sorting.

**[0165]** After several rounds of FACS sorting the final enriched population was plated out on SDCAA plates and incubated at 30°C for 2 days. Individual colonies were picked using a Genetix Clonepix and re-arrayed into 96 well plates containing SD-CAA medium. After incubation for 24 hours the cells were collected by centrifugation and resuspended in SD-GAA medium for induction of surface expressed unique Fn3 molecules. Positive clones were identified by standard ELISA. Plasmid DNA corresponding to the unique Fn3 positive clones was purified and sequenced to identify monospecific binders.

**[0166]** Once monospecific binders were identified and selected from either libraries A or B, they can be used independently to generate therapeutic molecules. In particular, the monospecific binders generated from library B using the bottom loops of the Fn3 molecule can be used to generate novel therapeutic binding molecules against a target of interest. Various monospecific binders from library B can be combined with linkers to produce a Fn3 binding molecule that is capable of binding to one or more regions of a single target (e.g., TNF). Alternately, a Fn3 binding molecule comprising binders from Library B can also be designed to bind to one or more regions of multiple targets (e.g., one or more regions of HSA and TNF).

**[0167]** In addition, the monospecific binders generated from Library A and Library B can be combined using standard molecular biology technique to generate bispecific and multispecific binders as described in Example 3 below.

### **Example 3**

#### Generation of Bifunctional Fibronectin-Based Binding Molecules

**[0168]** Computer modeling of the randomized regions onto the x-ray structure of human Fn3 shows that by combining

monospecific binders of each of the libraries A and B, one can create a bispecific fibronectin binding molecules. These binding molecules can be engineered such that they recognizes different regions on the same target molecule, or that the different binding sites of the bispecific or multispecific fibronectin molecule can bind to different regions on two or more different targets.

5 [0169] For example, a suitable sequence corresponding to binder A (obtained by screening Library A) and a suitable sequence corresponding to binder B (obtained by screening library B) can be combined into one single molecule to generate a bispecific molecule.

[0170] Binder A identified by screening library A (beta-sandwich with solvent exposed top loops BC, DE and FG).

10 VSDVPRDLEVVAATPTSLLISWXXXXXXXXRYRITYGETGGNSPVQEFTVPXXX  
XTATISGLKPGVDYITVYAVTXXXXXXXXXXXXPISINYRTEI (SEQ ID NO: 4)

15 where X is any amino acid sequence.

[0171] Binder B identified by screening library B (beta-sandwich with solvent exposed bottom loops AB, CD, EF and C-terminus).

20 VSDVPRDLEVVAATZZSLLISWDAPAVTVRYRITYGZZZZZZVQEFTVPGSKST  
ATZZZZZGZDYITVYAVTGRGDSPASSKPISINYZZZZ (SEQ ID NO: 5)

where Z is any amino acid sequence.

25 [0172] Binder C identified by combining sequences obtained by screening library A (beta-sandwich with solvent exposed top loops BC, DE and FG) and B (beta-sandwich with solvent exposed top loops BC, DE and FG). Merging of the two sequences into one molecule leads to the bispecific Binder C.

30 VSDVPRDLEVVAATZZSLLISWXXXXXXXXRYRITYGZZZZZZVQEFTVPXXX  
TATZZZZZGZDYITVYAVTXXXXXXXXXXXXPISINYZZZZ (SEQ ID NO: 6)

Where X and Z are any amino acid sequences.

35 [0173] The DNA corresponding to the combined amino acid sequence of Binder C is then synthesized and optimised for expression in *E.coli* at Genart AG, Germany. Cloning into *E.coli* and subsequent purification follows standard protocols as outlined in chapter 4 (Methods of manufacture).

[0174] Alternatively, a bifunctional Fn3-based binding molecule can be generated by linking two or more monospecific Fn3-based binding molecules.

40

#### **Example 4**

##### Generation of Monospecific Fibronectin-Based Binding Molecules that use the Bottom Loops to Bind a Target

45 [0175] The experimental details described in Example 2 were repeated to generate monospecific Fn3-based binding molecules that use the bottom loops of fibronectin to bind to the half-life extender, HSA. A separate library was used to generate monospecific Fn3-based binding molecules that use the bottom loops to bind to lysozyme.

##### *(a) Isolating of the enriched pool from yeast*

50

[0176] Sequences of selected populations were determined after several rounds of selection. Plasmid DNA was extracted from 1 mL of saturated culture of each selected yeast population using a Zymoprep kit (Zymo Research, Orange, CA), and 2 µL of the plasmid was transformed into electro-competent Top 10 *E. coli* (Invitrogen, Carlsbad, CA). Plasmid minipreps were prepared and sequenced for 96 colonies.

55

##### *(b) Sub-cloning into E. coli*

[0177] The genes encoding the enriched fibronectin pools were cloned into pNAT40 vector shown in Figure 6 and the

constructs transformed into electro-competent Acella *E. coli* strain (Gaithersburg, MD). A number of clones were selected and sequenced. The amino acid sequences of selected clones of potential Fn3-based binders for HSA are shown in Figure 7, along with a consensus binding sequence (SEQ ID NO: 42). Figure 8 shows the amino acid sequences of selected clones of potential Fn3-based binders for lysozyme.

**[0178]** Individual clones from the HSA library were selected and used to inoculate 1 ml of OvernightExpress media (Novagen, Gibbstown, NJ) containing 100 µg/ml Kanamycin in a 96 deep-well plate using a colony picker (Genetix Clonepix2, Boston, MA) and grown overnight at 37°C with vigorous shaking. The following day the cell pellets were harvested by centrifugation at 3000 rpm for 15 min and the supernatant discarded. The deep-well plates were frozen twice for 30 min at -80 °C and thawed at room temperature. Subsequently, 250 µl lysis-buffer containing TBS with 0.2 mg/ml lysozyme and 1 mM EDTA was added and the plates incubated at room temperature for 1 hour followed by the addition of 250 µl TBS containing 0.1 mg/ml DNaseI, 10 mM MgCl<sub>2</sub> and incubation for another 1 hour at room temperature. **[0179]** The lysed cell suspension was transferred onto 96 well Multiscreen HTS (Millipore, Billerica, MA) and the supernatant cleared by centrifugation. The cleared supernatant containing the potential Fn3-based binders for HSA was subsequently used for ELISA assay as described below in Example 5.

### **Example 5**

#### Binding Characterization of Monospecific Fibronectin-Based Binding Molecules

**[0180]** This Example illustrates, for the first time, that Fn3-based binding molecules can be generated by modifying at least one of the bottom loops of Fn3 (e.g., loops AB, CD and/or EF), to successfully produce Fn3-based molecules that bind to a target using the bottom loops. In this Example, the potential Fn3-based binders for HSA were examined.

**[0181]** To evaluate the binding characteristics, ELISA assays were used. Each well of a pretreated ELISA plate (Nunc Maxisorb) was coated with 100 µl of 1.0 µg/mL HSA in PBS covered with plastic film and incubated at 4°C overnight. The next day the coating solution was removed and the plates washed twice with PBS. The wells were blocked by adding 200 µL 25 mg/ml Casein/PBS per well and incubated for 1 hour at 37°C, in a moist sealed container. After removing of the blocking solution and washing twice with PBS 50 µl of the previously prepared *E. coli* lysate expressing potential monospecific binders was added and incubated with shaking for 1 hour. Subsequently the plates were washed 5 times with PBS/Tween 20 (0.05 %) buffer followed by the addition of HRP conjugated anti-His-tag antibody (Abcam, Cambridge, MA) preparation diluted in 0.25% casein/PBS, 50 µL per well. After incubation for 1 hour, the His-tag antibody solution was removed and the plates washed 5 times with PBS/Tween 20 (0.05%). Finally the ELISA was developed by adding substrate solution (SureBlue TMB microwell substrate solution, KPL) 50 µL per well. Positive clones were selected and subsequently expression screened to identify candidates for scale-up production.

**[0182]** Figure 9 shows representative, non-limiting examples of a human serum albumin-specific ELISA analysis of His-tagged fibronectin protein from selected clones. Crude lysates of soluble fibronectin Fn10 domains were added to wells of an ELISA plate, which were coated with HSA antigen and additionally blocked with PBS+1% casein. Detection of Fn3-based binders for HSA was performed using a monoclonal HRP conjugated anti-His antibody. The ELISA was developed by a TMB-substrate as described above. The OD-values (Y-axis) were measured at 450 nm by an ELISA-reader. Each bar represents an individual clone extract. The data shows a number of clones that are positive Fn3-based binders for HSA displaying binding that is about 3-fold higher than the background. The results demonstrate, for the first time, that the bottom loops of the fibronectin molecule can be used to bind to a target, such as HSA.

**[0183]** To purify the expressed monospecific Fn3-based binders for HSA, the cleared lysate was transferred into a 96 well deep well block and 20 µL of a 50% (v/v) slurry of Ni<sup>2+</sup>-loaded MagneHis beads (Promega) added. Automated purification was performed using a KingFisher Robot (Thermo Scientific). After a 30 min incubation the beads were washed twice with 1 ml wash buffer/well (50 mM Phosphate, 1 M NaCl, 20 mM Imidazole, pH 7.6). Finally bound fibronectin protein was eluted with 200 µl of PBS + 300 mM Imidazole at pH 7.6. Aliquots of the crude extract and elute were analyzed by SDS gel-electrophoresis (data not shown).

**[0184]** To further demonstrate that the bottom side Fn3-based binders for HSA can be successfully expressed, a small-scale total expression experiment was conducted in *E. coli*. Samples from total expression of 24 constructs were analyzed on a Novex BisTris 4-2% NuPAGE gel, 26 wells (Invitrogen). Lane 1 contains size marker SeeBlue plus2 (Invitrogen). The results shows successful expression of fibronectin molecules with modified bottom loops in *E. coli*. A protein band at about 16kDa (corresponding to fibronectin Fn10 (~ 11-12kDa) plus N-terminal His-tag, S-tag and pre-scission cleavage site) appears in a majority of the selected clones, as shown in Figure 10. These results further demonstrate that the monospecific Fn3-based binding molecule can successfully be expressed in *E. coli* and retain binding activity.

**[0185]** To show that the expressed proteins can be purified without adverse effect on the protein structure (e.g., degradation), the samples were purified as described above and analyzed on a Novex BisTris 4-12% NuPAGE gel, 26 wells (Invitrogen). Lane 1 contains size marker Mark 12 (Invitrogen). The 16 kDa band clearly appears in most of the

## EP 2 274 331 B1

clones, as shown in Figure 10. Mass spectrometry analysis confirms the presence of fibronectin at the correct molecular weight being purified, as shown in Table 1.

Table 1: Mass Spectrometry Analysis of Fn3-based Fibronectin Binding Molecules

Clone number	Molecular Weight
Clone 1	16076
Clone 2	16265
Clone 3	12112
Clone 4	ND
Clone 5	16395
Clone 6	ND
Clone 7	16274
Clone 8	16321
Clone 9	ND
Clone 10	ND
Clone 11	ND
Clone 12	16274
Clone 13	16394
Clone 14	ND
Clone 15	ND
Clone 16	ND
Clone 17	ND
Clone 18	16482
Clone 19	16275
Clone 20	16380
Clone 21	16274
Clone 22	18319
Clone 23	ND
Clone 24	ND
ND = not determined	

**[0186]** Collectively, the results show for the first time that Fn3 -based binding molecules can be generated in which at least one of the bottom loops is modified such that it binds to a target. To date, the top loops have typically been analyzed for binding to targets, primarily due to the better alignment of the top loops with antibody sequences compared with the bottom loops. The results presented herein demonstrate that the bottom loops can be modified without adversely affecting the stability of the molecule. Thus, a library of Fn3 -based binding molecules can be generated in which at least one of the bottom loops is modified such that it binds to a target. With the HSA library, the bottom loops were kept at the same length as wild type Fn3 and modified. With the lysozyme library, the bottom loops were varied in length compared with wild type Fn3 without adverse effects on the protein structure. Furthermore, these monospecific Fn3 -based binding molecules with the modified bottom loops, maintain conformational stability and can be expressed and purified while retaining binding ability. Thus, the methods of the invention can be used to generate a library of Fn3-based binding molecules that have at least one modified bottom loop as well as Fn3-based binding molecules that use the bottom face to bind a target.

**Example 6**Generation and Characterization of Multispecific Fibronectin-Based Binding Molecules

5 **[0187]** This Example demonstrates the production and characterization of a multispecific Fn3-based binding molecule, in which two separate monospecific Fn3-based binding molecules are linked together in a pearl-like fashion, using a linker sequence to generate a multispecific, e.g., bispecific molecule. The bispecific molecule was designed as follows. The VEGFR2 binding sequence shown below (SEQ ID NO: 117) was fused to the identified bottom side HSA binder, identified in Figure 7 and shown below (SEQ ID NO: 8), using a short GS linker sequence GGGGSGGGGS (SEQ ID NO: 118), to generate a multispecific Fn3-based binding molecule with SEQ ID NO: 119, shown below. The constructs were synthesized by DNA2.0 and sub-cloned into expression vector pJExpress 401. Cloning, purification and ELISA followed standard protocols as outlined above.

15 *VEGFR top side sequence*

**[0188]**

20 MGVS DVPRDLEVVAATPTSLLISWRHPHPTRYRITYGETGGNSPVQEF  
TVPLQPPLATISGLKPGVDYTITVYAVTKERNGRELFTPI SINYRT (SEQ ID NO:  
117)

25 *Bottom side sequence 8 from Figure 7*

**[0189]**

30 MGVS DVPRDLEVVAATPTSLLISWDAPAVTVRYRITYGR TDAKSTRKE  
FTVPGSKSTATIGELKRGRDYTITVYAVTGRGDSPASSKPI SINYRPEK (SEQ ID  
NO: 8)

35 *VEGFR2-Bottom sequence 8 from Figure 7 fused with GS linker (clone 87)*

**[0190]**

40 MGVS DVPRDLEVVAATPTSLLISWRHPHPTRYRITYGETGGNSPVQEF  
TVPLQPPLATISGLKPGVDYTITVYAVTKERNGRELFTPI SINYRTGGGGSGGGGS  
VSDVPRDLEVVAATPTSLLISWDAPAVTVRYRITYGR TDAKSTRKEFTVPGSK  
STATIGELKRGRDYTITVYAVTGRGDSPASSKPI SINYRPEKENLYFQGH HHHHHH  
45 (SEQ ID NO: 119)

**[0191]** The ELISA binding studies of the multispecific molecule is shown in Figure 13 and discussed in more detail in Example 7.

50 **[0192]** To demonstrate that the multispecific Fn3-based binding molecule can be purified from a large scale expression of the molecule, the procedure outlined by Gräslund *et al.*, was used (Gräslund *et al.* Nature Methods (2008) 5, 135 - 146). Starter cultures of 10 ml (~1/100 of the final cultivation volume) were used to inoculate Minimal Media containing 25 ug/ml Kanamycin and incubated overnight at 37°C at 175 rpm. The following morning, 10 ml of the starter culture was used to inoculate into 1 litre of OvernightExpress media with 100 ug/ml Kanamycin in a 2.8 L Fernbach shake flask. The cells were incubated at 37°C at 175 rpm until an optical density at 600 nm (OD600) of 1-1.5 was reached. The temperature of the culture was then lowered to 18°C to switch on expression and the cultures maintained overnight at 18°C for continued expression. The cells were harvested by centrifugation at 4500 x g for 10 min. The cells were weighed, and 1 volume (v/v) of lysis buffer (0.1 M sodium phosphate, pH 8.0, 1.0 M NaCl, 20 mM imidazole, 10% (v/v)

## EP 2 274 331 B1

glycerol, 1 mM TCEP, and 20 units/ml Benzonase) was added. 1 mg/ml lysozyme was added to the cell suspension and after incubation for 30 min on ice, the suspension sonicated intermittently for 1-2 minutes. The lysate was diluted with 2-3 volumes of Lysis buffer and 1 ml Ni-NTA resin was added. Binding was performed by slowly rotating the supernatant in a 250 ml conical vial at 4°C for 1 hour. The resin was collected in a 20 ml disposable column and washed with loading buffer (0.05 M sodium phosphate, pH 8.0, 0.5 M NaCl, 20 mM imidazole, 5% (v/v) glycerol, 0.5 mM TCEP) until no more protein eluted (20-30 column volumes of each). The bound protein was eluted with elution buffer (0.05 M sodium phosphate, pH 8.0, 0.5 M NaCl, 0.3 M imidazole, 5% (v/v) glycerol, 0.5 mM TCEP). Peak fractions were identified, pooled together and loaded onto an equilibrated MonoS 5/50 GL column (GE) with buffer S (50 mM Hepes, 50 mM NaCl pH 7.6). The Ni-NTA elute was diluted 10 fold with buffer S and load onto the pre-equilibrated column. After washing with 10 volumes of Buffer S the protein was eluted with a linear gradient from 50 mM to 1 M NaCl in buffer S. Fractions were analysed by SDS-PAGE and mass spectrometry.

**[0193]** Fig. 12 The left panel shows the SDS-PAGE of the different purification steps of clone 87. Lane 1, total protein; lane 2, crude extract; lane 3, Ni-NTA flow through; lane 4, Ni-NTA wash; lane 5, Ni-NTA, elute; lane 6, Mono S elute. The right panel shows the mass spectrum of the purified sample (MW 23404 D) consistent with the expected mass of the N-terminal methionine cleaved protein of clone 87.

### Example 7

#### Generation and Characterization of Bispecific Fibronectin-Based Binding Molecules

**[0194]** This Example demonstrates the production and characterization of a bispecific Fn3-based binding molecule in which a single fibronectin molecule has both the top loops and the bottom loops modified such that the single fibronectin molecule can bind two separate targets, e.g., VEGFR2 and HSA.

**[0195]** A bispecific molecule was generated by crafting the bottom side HSA binding loop sequence onto the top-side VEGFR2 binders generating a molecule with bispecific functionality in which both the top and bottom loops of the same Fn3 molecule can be used to bind to two different target molecules. To create the bispecific molecule, the bottom side binder (SEQ ID NO: 8) loops (shown above) were crafted onto the VEGFR2 binder (SEQ ID NO: 117) (shown above) by excising the bottom loops and inserting them into the VEGFR2 binder, yielding molecule VEGFR2-HAS (SEQ ID NO: 120). The constructs were synthesized by DNA2.0 and sub-cloned into expression vector pJExpress 401. Cloning, purification and ELISA followed standard protocols as outlined above.

*VEGFR2/Bottom side SEQ ID NO: 8 merged molecule (clone 89)*

**[0196]**

MGVSDVPRDLEVVAATPTSLISWRHPHFTRYRITYGRDPAKSTRKEF  
TVPLQPPLATIGELKRGDYYTITVYAVTKERNGRELFTPIISINYRPEKENLYFQGH  
HHHHH (SEQ ID NO: 120)

*(a) ELISA for dual-specific fibronectin binding molecules from Examples 6 and 7:*

**[0197]** The binding characteristic of the multispecific and bispecific binders from Examples 6 and 7 were assessed as follows. VEGFR2-Fc fusion protein was immobilized onto a pretreated ELISA plate (NuncMultisorb) and additionally blocked with PBS+1%Casein. Aliquots of cleared lysate of wild-type Fin10 (lane1), clone 87 (multispecific) (lane2), clone 89 (bispecific) (lane3) and PBS were added to the wells and incubated for 1 hour at room-temperature. These were run at different concentrations 1, 2 and 3. After washing 5 times with PBS/0.05% Tween, the wells were incubated with 1 µg/ml HSA in PBS. Detection was performed with HRP conjugated chicken anti-human HSA antibody (Abcam). The ELISA was developed with a TMP substrate and the OD-values measured at 450 nm. The results are shown in Figure 13.

**[0198]** Consistent reproducible results were obtained with both multispecific and bispecific Fn3-based binding molecules present in crude extract, showing that clone 87 (multispecific) and clone 89 (bispecific) bind to both HSA and VEGFR2. This data demonstrates that multispecific and bispecific Fn3 molecules can be produced where one of the monospecific fibronectin binding molecules has at least one modified bottom loop (AB, DE, FG) that can bind HSA (see Figure 13).

**[0199]** The multispecific Fn3 molecule (clone 87) has two separate monospecific Fn3 molecules linked together via a G-S linker. One monospecific Fn3 molecule has modified bottom loops that bind to HSA while the other monospecific Fn3 molecule has modified top loops that bind to VEGFR2. The resulting multispecific molecule retains the ability to

## EP 2 274 331 B1

bind to two different targets using each of the monospecific fibronectin molecules. This data shows that monospecific Fn3 molecules can be linked together in a pearl-like manner to create a multispecific Fn3 molecule; that the linker does not interfere with the binding ability of each of the monomeric Fn3 molecules; and that the multispecific Fn3 molecule is able to bind to two different targets without any steric hinderance issues.

5 [0200] The bispecific fibronectin molecule (clone 89) is a single fibronectin molecule engineered such that the bottom loops bind to HSA and the top loops bind to VEGFR2. The single bispecific fibronectin molecule successfully binds both HSA and VEGFR2 Figure 13 shows that the single bispecific fibronectin molecule is able to bind both HSA and VEGFR2 without any steric hinderance issues. This data shows, for the first time, that bispecific fibronectin molecules can be produced; that the binding ability of the loops on each side of the bispecific fibronectin molecule is retained; that the structural confirmation of the bispecific molecule is maintained with both bottom and top loops being modified at the same time; that the top and bottom loops function independently of each other to bind separate targets; and that the bispecific Fn3 molecule is able to bind to two different targets without any steric hinderance issues.

15 (b) *Biacore for dual-specific fibronectin binding molecules from Examples 6 and 7:*

20 [0201] The binding kinetics of fibronectin-based scaffold proteins binding proteins to the target was measured using BIAcore T100 (GE). Anti-hu IgG (GE, human IgG capture kit) Fc specific antibody was immobilized onto a CM5 sensor chip and soluble VEGFR2-huFc fusion protein captured on the surface. Soluble Fibronectin clone 87 (multispecific) was injected at 100 nM (top line) and 200 nM (bottom line) in buffer 0 (10 mM HEPES, 150 mM NaCl, 0.005% Tween 20, pH 7.4). Sensorgrams were obtained at each concentration and the rate constants  $k_a$  ( $k_{on}$ ) and  $k_d$  ( $k_{off}$ ) determined. The affinity constant, KD was calculated from the ratio of rate constants  $k_{off}/k_{on}$ .

25 [0202] The results for clone 87 are shown in Figures 14 and 15 for the top loop binding VEGFR2 and the bottom loop binding HSA, respectively.. This data shows the multispecific fibronectin comprising two separate monospecific Fn3 molecules linked together with a G-S linker, successfully binds to two separate target molecules using the bottom and top loops.

[0203] The same experiment was repeated for clone 89, the single bispecific Fn3 molecule and similar results obtained (data not shown).

30 [0204] Collectively, these results show for the first time that multispecific and bispecific fibronectin molecules can be produced. These molecules were successfully expressed in *E. coli*, were be purified without degradation, retained binding ability, and were able to bind to two separate targets.

### Equivalents

35 [0205] Those skilled in the art will recognize or be able to ascertain, using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

## IMPROVED FIBRONECTIN-BASED BINDING MOLECULES AND THEIR USE

### Abstract of Disclosure

40 [0206] The invention provides bispecific Fn3-based binding molecules that bind to two or more targets simultaneously. The Fn3-based binding molecules of the invention can also be linked together to form multispecific Fn3-based binding molecules, and/or can be conjugated to a non-Fn3 moiety, such as, Human Serum Albumin (HSA), for improved half life and stability. The invention also provides methods for generating, screening and using Fn3-based binding molecules in a variety of therapeutic and diagnostic applications.

### Claims

- 50
1. A bispecific Fn3-based binding molecule comprising an Fn3 domain, wherein at least one amino acid in one or more of the bottom AB, CD or EF loop regions or C-terminal of the Fn3 domain are altered compared to the wild-type Fn3 domain comprising SEQ ID NO:1 to create a non-Fn3 binding sequence which binds to a first target, and wherein at least one amino acid in one or more of the top BC, DE or FG loop regions of the Fn3 domain are altered compared to the wild-type Fn3 domain comprising SEQ ID NO:1 to create a non-Fn3 binding sequence which binds to a second target.
  2. The bispecific Fn3-based binding molecule of claim 1, wherein said at least one amino acid in said bottom AB, CD
- 55

or EF loop regions is selected from the group consisting of the amino acids at position 15, 16, 38, 39, 40, 41, 42, 43, 44, 45, 60, 61, 62, 63, 64, 93, 95, and 96 of SEQ ID NO:1, and wherein said at least one amino acid in said top BC, DE or FG loop regions is selected from the group consisting of amino acids at position 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 51, 52, 53, 54, 55, 56, 76, 77, 78, 79, 80, 80, 81, 82, 83, 84, 85, 86, 87, and 88 of SEQ ID NO:1.

- 5
3. The bispecific Fn3-based binding molecule of claims 1 or 2, wherein the non-Fn3 binding sequence comprises all or a portion of a complementarity determining region (CDR) of an antibody or a T-cell receptor.
- 10
4. The bispecific Fn3-based binding molecule of any of claims 1 to 3, wherein said first and second targets are present on the same molecule.
5. The bispecific Fn3-based binding molecule of claim 1 to 2, wherein said first and second targets are present on different molecules.
- 15
6. A bispecific Fn3-based binding molecule of claims 1 to 3 or 5, wherein the first target is a half-life extender.
7. The bispecific Fn3-based binding molecule of claim 6, wherein the half-life extender is human serum albumin.
8. The bispecific Fn3-based binding molecule of claim 6, wherein the second target is VEGFR2.
- 20
9. The Fn3-based binding molecule of any of the preceding claims, which binds to a non-Fn3 moiety that increases the half-life of the Fn3-based binding molecule.
- 25
10. A multispecific Fn3-based binding molecule comprising two or more monospecific Fn3-based binding molecules, wherein at least one monospecific Fn3-based binding molecule comprises at least one amino acid in one or more of the bottom AB, CD or EF loop regions or C-terminal of the Fn3 domain that is altered compared to the wild-type Fn3 domain comprising SEQ ID NO: 1 to create a non-Fn3 binding sequence which binds to a first target; and a second monospecific Fn3-based binding molecule having at least one amino acid in one or more of the top BC, DE or FG loop regions of the Fn3 domain that is altered compared to the wild-type Fn3 domain comprising SEQ ID NO: 1 to create a non-Fn3 binding sequence which binds to a second target, wherein the first and second monospecific Fn3-based binding molecules are connected by a linker sequence.
- 30
11. The multispecific Fn3-based binding molecule of claim 10, further comprising a third monospecific Fn3-based binding molecule having at least one amino acid in one or more of the top BC, DE or FG loop regions of the Fn3 domain that is altered compared to the wild-type Fn3 domain comprising SEQ ID NO:1 to create a non-Fn3 binding sequence which binds to one or more targets, and wherein the second and third monospecific Fn3-based binding molecules are connected by a linker sequence.
- 35
12. The multispecific Fn3-based binding molecule of claim 10, wherein the first target is a half-life extender.
- 40
13. The multispecific Fn3-based binding molecule of claim 12, wherein the half-life extender is human serum albumin.
14. The multispecific Fn3-based binding molecule of claim 12, wherein the second target is VEGFR2.
- 45
15. The multispecific Fn3-based binding molecule of claim 12, wherein the linker sequence is a G-S linker sequence.
16. A bispecific Fn3-based binding molecule comprising SEQ ID NO: 120.
- 50
17. A conjugate comprising an Fn3-based binding molecule of any of the preceding claims linked to one or more non-Fn3 moieties.
18. The conjugate of claim 17, wherein the non-Fn3 moiety comprises or binds to a molecule which increases the half-life of the Fn3-based binding molecule.
- 55
19. The Fn3-based binding molecule or conjugate of claims 17-18, wherein the non-Fn3 moiety comprises a molecule selected from the group consisting of an antibody Fc region, Human Serum Albumin (HSA) and polyethylene glycol (PEG).

20. The Fn3-based binding molecule or conjugate of any of the preceding claims which further comprises at least one modified amino acid residue compared to the wild-type Fn3 domain comprising SEQ ID NO: 1 for attaching a functional moiety.
- 5 21. The Fn3-based binding molecule or conjugate of claim 20, wherein the modified amino acid residue comprises the addition or substitution of a cysteine residue or non-natural amino acid residue.
22. The Fn3-based binding molecule or conjugate of any of the preceding claims, comprising beta strands from two or more different Fn3 domains.
- 10 23. A composition comprising the Fn3-based binding molecule or conjugate of any of the preceding claims and a carrier.
24. A composition comprising the Fn3-based binding molecule or conjugate of any of the preceding claims and a carrier for use as a medicament.
- 15 25. A composition for use as a medicament according to claim 24, wherein the medicament is used to treat a subject for a disease selected from the group consisting of an autoimmune disease, a cancer, and an infectious disease.
- 20 26. A method of detecting a protein in a sample comprising labeling the Fn-3 based binding molecule or conjugate of any of the preceding claims, contacting the labeled binding molecule or conjugate with the sample, and detecting complex formation between the Fn3-based binding molecule or conjugate with the protein.
27. A variegated nucleic acid library encoding Fn3-based binding molecules of any one of claims 6 and 10.

25

#### Patentansprüche

- 30 1. Bispezifisches Fn3-basiertes Bindemolekül, umfassend eine Fn3-Domäne, wobei zumindest eine Aminosäure in einer oder mehreren der unteren AB-, CD- oder EP-Loop-Regionen oder C-Terminal der Fn3-Domäne im Vergleich zur Wildtyp-Fn3-Domäne, umfassend SEQ ID Nr. 1, verändert ist, um eine Nicht-Fn3-Bindesequenz zu bilden, die an ein erstes Ziel bindet, und wobei zumindest eine Aminosäure in einer oder mehreren der oberen BC-, DE- oder FG-Loop-Regionen der Fn3-Domäne im Vergleich zur Wildtyp-Fn3-Domäne, umfassend SEQ ID Nr. 1, verändert ist, um eine Nicht-Fn3-Bindesequenz zu bilden, die an ein zweites Ziel bindet.
- 35 2. Das bispezifische Fn3-basierte Bindemolekül nach Anspruch 1, wobei diese zumindest eine Aminosäure in den unteren AB-, CD- oder EF-Loop-Regionen ausgewählt ist aus der Gruppe bestehend aus den Aminosäuren an Position 15, 16, 38, 39, 40, 41, 42, 43, 44, 45, 60, 61, 62, 63, 64, 93, 95 und 96 von SEQ ID Nr. 1, und wobei diese zumindest eine Aminosäure in diesen oberen BC-, DE- oder FG-Loop-Regionen ausgewählt ist aus der Gruppe bestehend aus Aminosäuren an Position 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 51, 52, 53, 54, 55, 56, 76, 77, 78, 79, 80, 80, 81, 82, 83, 84, 85, 86, 87 und 88 von SEQ ID Nr. 1.
- 40 3. Das bispezifische Fn3-basierte Bindemolekül nach Anspruch 1 oder 2, wobei die Nicht-Fn3-Bindesequenz die ganze oder einen Teil einer komplementaritätsbestimmenden Region (CDR) eines Antikörpers oder eines T-Zellen-Rezeptors umfasst.
- 45 4. Das bispezifische Fn3-basierte Bindemolekül nach einem der Ansprüche 1 bis 3, wobei dieses erste und zweite Ziel auf dem gleichen Molekül vorhanden sind.
- 50 5. Das bispezifische Fn3-basierte Bindemolekül nach den Ansprüchen 1 bis 2, wobei dieses erste und zweite Ziel auf unterschiedlichen Molekülen vorhanden sind.
6. Bispezifisches Fn3-basiertes Bindemolekül nach den Ansprüchen 1 bis 3 oder 5, wobei das erste Ziel ein Halbwertszeitverlängerer ist.
- 55 7. Das bispezifische Fn3-basierte Bindemolekül nach Anspruch 6, wobei der Halbwertszeitverlängerer humanes Serumalbumin ist.
8. Das bispezifische Fn3-basierte Bindemolekül nach Anspruch 6, wobei das zweite Ziel VEGFR2 ist.

9. Das Fn3-basierte Bindemolekül nach einem der vorstehenden Ansprüche, das an eine Nicht-Fn3-Gruppe bindet, der die Halbwertszeit des Fn3-basierten Bindemoleküls erhöht.
- 5 10. Multispezifisches Fn3-basiertes Bindemolekül, umfassend zwei oder mehrere monospezifische Fn3-basierte Bindemoleküle, wobei zumindest ein monospezifisches Fn3-basiertes Bindemolekül zumindest eine Aminosäure in einer oder mehreren der unteren AB-, CD- oder EF-Loop-Regionen oder C-Terminal der Fn3-Domäne umfasst, die im Vergleich zur Wildtyp-Fn3-Domäne, umfassend SEQ ID Nr. 1, verändert ist, um eine Nicht-Fn3-Bindesequenz zu bilden, die an ein erstes Ziel bindet; und ein zweites monospezifisches Fn3-basiertes Bindemolekül mit zumindest einer Aminosäure in einer oder mehreren der oberen BC-, DE- oder FG-Loop-Regionen der Fn3-Domäne, die im Vergleich zur Wildtyp-Fn3-Domäne, umfassend SEQ ID Nr. 1, verändert ist, um eine Nicht-Fn3-Bindesequenz zu bilden, die an ein zweites Ziel bindet, wobei das erste und zweite monospezifische Fn3-basierte Bindemolekül durch eine Linkersequenz verbunden sind.
- 10 11. Das multispezifische Fn3-basierte Bindemolekül nach Anspruch 10, ferner umfassend ein drittes monospezifisches Fn3-basiertes Bindemolekül mit zumindest einer Aminosäure in einer oder mehreren der oberen BC-, DE- oder FG-Loop-Regionen der Fn3-Domäne, die im Vergleich zur Wildtyp-Fn3-Domäne, umfassend SEQ ID Nr. 1, verändert ist, um eine Nicht-Fn3-Bindesequenz zu bilden, die an ein oder mehrere Ziele bindet, und wobei das zweite und dritte monospezifische Fn3-basierte Bindemolekül durch eine Linkersequenz verbunden sind.
- 15 12. Das multispezifische Fn3-basierte Bindemolekül nach Anspruch 10, wobei das erste Ziel ein Halbwertszeitverlängerer ist.
- 20 13. Das multispezifische Fn3-basierte Bindemolekül nach Anspruch 12, wobei der Halbwertszeitverlängerer humanes Serumalbumin ist.
- 25 14. Das multispezifische Fn3-basierte Bindemolekül nach Anspruch 12, wobei das zweite Ziel VEGFR2 ist.
15. Das multispezifische Fn3-basierte Bindemolekül nach Anspruch 12, wobei die Linkersequenz eine G-S-Linkersequenz ist.
- 30 16. Bispezifisches Fn3-basiertes Bindemolekül, umfassend SEQ ID Nr. 120.
17. Konjugat, umfassend ein Fn3-basiertes Bindemolekül nach einem der vorstehenden Ansprüche, das mit einer oder mehreren Nicht-Fn3-Gruppe(n) verknüpft ist.
- 35 18. Das Konjugat nach Anspruch 17, wobei der Nicht-Fn3-Gruppe ein Molekül umfasst oder an dieses bindet, das die Halbwertszeit des Fn3-basierten Bindemoleküls erhöht.
19. Das Fn3-basierte Bindemolekül oder Konjugat nach den Ansprüchen 17 bis 18, wobei die Nicht-Fn3-Gruppe ein Molekül umfasst, das aus der Gruppe bestehend aus einer Antikörper-Fc-Region, humanem Serumalbumin (HSA) und Polyethylenglykol (PEG) ausgewählt ist.
- 40 20. Das Fn3-basierte Bindemolekül oder Konjugat nach einem der vorstehenden Ansprüche, das ferner zumindest einen modifizierten Aminosäurerest im Vergleich zur Wildtyp-Fn3-Domäne, umfassend SEQ ID Nr. 1, zur Anhaftung einer funktionellen Gruppe umfasst.
- 45 21. Das Fn3-basierte Bindemolekül oder Konjugat nach Anspruch 20, wobei der modifizierte Aminosäurerest die Addition oder Substitution eines Cysteinrests oder eines nichtnatürlichen Aminosäurerests umfasst.
- 50 22. Das Fn3-basierte Bindemolekül oder Konjugat nach einem der vorstehenden Ansprüche, umfassend beta-Stränge aus zwei oder mehreren unterschiedlichen Fn3-Domänen.
23. Zusammensetzung, umfassend das Fn3-basierte Bindemolekül oder Konjugat nach einem der vorstehenden Ansprüche und einen Träger.
- 55 24. Zusammensetzung, umfassend das Fn3-basierte Bindemolekül oder Konjugat nach einem der vorstehenden Ansprüche und einen Träger zur Verwendung als Medikament.

## EP 2 274 331 B1

25. Zusammensetzung zur Verwendung als Medikament nach Anspruch 24, wobei das Medikament verwendet wird, um ein Individuum wegen einer Krankheit zu behandeln, die aus der Gruppe bestehend aus einer Autoimmunerkrankung, Krebs und einer Infektionskrankheit ausgewählt ist.
- 5 26. Verfahren zum Nachweisen eines Proteins in einer Probe, umfassend das Markieren des Fn3-basierten Bindemoleküls oder Konjugats nach einem der vorstehenden Ansprüche, Inkontaktbringen des markierten Bindemoleküls oder Konjugats mit der Probe und Nachweisen einer Komplexbildung zwischen dem Fn3-basierten Bindemolekül oder Konjugat und dem Protein.
- 10 27. Vielfältige Nukleinsäurebibliothek, die Fn3-basierte Bindemoleküle nach einem der Ansprüche 6 und 10 kodiert.

### Revendications

- 15 1. Molécule de liaison à base de Fn3 bispécifique comprenant un domaine Fn3, dans laquelle au moins un acide aminé dans une ou plusieurs des régions en boucle AB, CD ou EF inférieures ou de l'extrémité C terminale du domaine Fn3 étant altéré par comparaison au domaine Fn3 de type sauvage comprenant SEQ ID NO:1 pour créer une séquence de liaison à non-Fn3 qui se lie à une première cible, et dans laquelle au moins un acide aminé dans une ou plusieurs des régions en boucle BC, DE ou FG supérieures du domaine Fn3 est altéré par comparaison au domaine Fn3 de type sauvage comprenant SEQ ID NO:1 pour créer une séquence de liaison à non-Fn3 qui se lie à une seconde cible.
- 20 2. Molécule de liaison à base de Fn3 bispécifique selon la revendication 1, dans laquelle ledit au moins un acide aminé dans lesdites régions en boucle AB, CD ou EF inférieures est choisi dans le groupe consistant en les acides aminés en position 15, 16, 38, 39, 40, 41, 42, 43, 44, 45, 60, 61, 62, 63, 64, 93, 95 et 96 de SEQ ID NO:1, et dans laquelle ledit au moins un acide aminé dans lesdites régions en boucle BC, DE ou FG supérieures est choisi dans le groupe consistant en acides aminés en position 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 51, 52, 53, 54, 55, 56, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87 et 88 de SEQ ID NO:1.
- 25 3. Molécule de liaison à base de Fn3 bispécifique selon l'une des revendications 1 ou 2, dans laquelle la séquence de liaison à non-Fn3 comprend tout ou partie d'une région de détermination de complémentarité (CDR) d'un anticorps ou d'un récepteur de lymphocytes T.
- 30 4. Molécule de liaison à base de Fn3 bispécifique selon l'une quelconque des revendications 1 à 3, dans laquelle lesdites première et seconde cibles sont présentes sur la même molécule.
- 35 5. Molécule de liaison à base de Fn3 bispécifique selon l'une des revendications 1 et 2, dans laquelle lesdites première et seconde cibles sont présentes sur des molécules différentes.
- 40 6. Molécule de liaison à base de Fn3 bispécifique selon l'une des revendications 1 à 3 ou 5, dans laquelle la première cible est un allongeur de demi-vie.
- 45 7. Molécule de liaison à base de Fn3 bispécifique selon la revendication 6, dans laquelle l'allongeur de demi-vie est le sérum albumine humaine.
8. Molécule de liaison à base de Fn3 bispécifique selon la revendication 6, dans laquelle la seconde cible est VEGFR2.
9. Molécule de liaison à base de Fn3 selon l'une quelconque des revendications précédentes, qui se lie à une fraction non-Fn3 qui augmente la demi-vie de la molécule de liaison à base de Fn3.
- 50 10. Molécule de liaison à base de Fn3 multi-spécifique comprenant au moins deux molécules de liaison à base de Fn3 monospécifiques, dans laquelle au moins une molécule de liaison à base de Fn3 monospécifique comprend au moins un acide aminé dans une ou plusieurs des régions en boucle AB, CD ou EF inférieures ou de l'extrémité C terminale du domaine Fn3 qui est altéré par comparaison au domaine Fn3 de type sauvage comprenant SEQ ID NO:1 pour créer une séquence de liaison à non-Fn3 qui se lie à une première cible ; et une deuxième molécule de liaison à base de Fn3 monospécifique ayant au moins un acide aminé dans une ou plusieurs des régions en boucle BC, DE ou FG supérieures du domaine Fn3 qui est altéré par comparaison au domaine Fn3 de type sauvage comprenant SEQ ID NO:1 pour créer une séquence de liaison à non-Fn3 qui se lie à une seconde cible, dans
- 55

## EP 2 274 331 B1

laquelle les première et deuxième molécules de liaison à base de Fn3 monospécifiques sont reliées par une séquence de liaison.

- 5
11. Molécule de liaison à base de Fn3 multi-spécifique selon la revendication 10, comprenant en outre une troisième molécule de liaison à base de Fn3 monospécifiques ayant au moins un acide aminé dans une ou plusieurs des régions en boucle BC, DE ou FG supérieures du domaine Fn3 qui est altéré par comparaison au domaine Fn3 de type sauvage comprenant SEQ ID NO:1 pour créer une séquence de liaison à non-Fn3 qui se lie à une ou plusieurs cibles, et dans laquelle les deuxième et troisième molécules de liaison à base de Fn3 monospécifiques sont reliées par une séquence de liaison.
- 10
12. Molécule de liaison à base de Fn3 multi-spécifique selon la revendication 10, dans laquelle la première cible est un allongeur de demi-vie.
13. Molécule de liaison à base de Fn3 multi-spécifique selon la revendication 12, dans laquelle l'allongeur de demi-vie est le sérum albumine humaine.
- 15
14. Molécule de liaison à base de Fn3 multi-spécifique selon la revendication 12, dans laquelle la seconde cible est VEGFR2.
- 20
15. Molécule de liaison à base de Fn3 multi-spécifique selon la revendication 12, dans laquelle la séquence de liaison est une séquence de liaison G-S.
16. Molécule de liaison à base de Fn3 bispécifique comprenant SEQ ID NO:120.
- 25
17. Conjugué comprenant une molécule de liaison à base de Fn3 selon l'une quelconque des revendications précédentes liée à une ou plusieurs fractions non-Fn3.
18. Conjugué selon la revendication 17, dans lequel la fraction non-Fn3 comprend ou se lie à une molécule qui augmente la demi-vie de la molécule de liaison à base de Fn3.
- 30
19. Molécule de liaison à base de Fn3 ou conjugué selon les revendications 17-18, dans laquelle ou dans lequel la fraction non-Fn3 comprend une molécule choisie dans le groupe consistant en une région Fc d'anticorps, la Sérum Albumine Humaine (HSA) et le polyéthylène glycol (PEG).
- 35
20. Molécule de liaison à base de Fn3 ou conjugué selon l'une quelconque des revendications précédentes, qui comprend en outre au moins un résidu d'acide aminé modifié par comparaison au domaine Fn3 de type sauvage comprenant SEQ ID NO:1 pour attacher une fraction fonctionnelle.
- 40
21. Molécule de liaison à base de Fn3 ou conjugué selon la revendication 20, dans laquelle ou dans lequel le résidu acide aminé modifié comprend l'addition ou la substitution d'un résidu cystéine ou d'un résidu d'acide aminé non naturel.
22. Molécule de liaison à base de Fn3 ou conjugué selon l'une quelconque des revendications précédentes, comprenant des brins bêta provenant d'au moins deux domaines Fn3 différents.
- 45
23. Composition comprenant la molécule de liaison à base de Fn3 ou conjugué selon l'une quelconque des revendications précédentes et un support.
24. Composition comprenant la molécule de liaison à base de Fn3 ou le conjugué selon l'une quelconque des revendications précédentes et un support, en vue de l'utilisation comme médicament.
- 50
25. Composition destinée à être utilisée comme médicament selon la revendication 24, dans laquelle le médicament est utilisé pour traiter un sujet pour une maladie choisie dans le groupe consistant en une maladie auto-immune, un cancer et une maladie infectieuse.
- 55
26. Procédé de détection d'une protéine dans un échantillon comprenant le marquage de la molécule de liaison à base de Fn3 ou du conjugué selon l'une quelconque des revendications précédentes, la mise en contact de la molécule de liaison marquée ou du conjugué marqué avec l'échantillon, et la détection de la formation d'un complexe entre

## EP 2 274 331 B1

la molécule de liaison à base de Fn3 ou le conjugué avec la protéine.

27. Bibliothèque d'acides nucléiques panachés codant pour des molécules de liaison à base de Fn3 selon l'une quelconque des revendications 6 et 10.

5

10

15

20

25

30

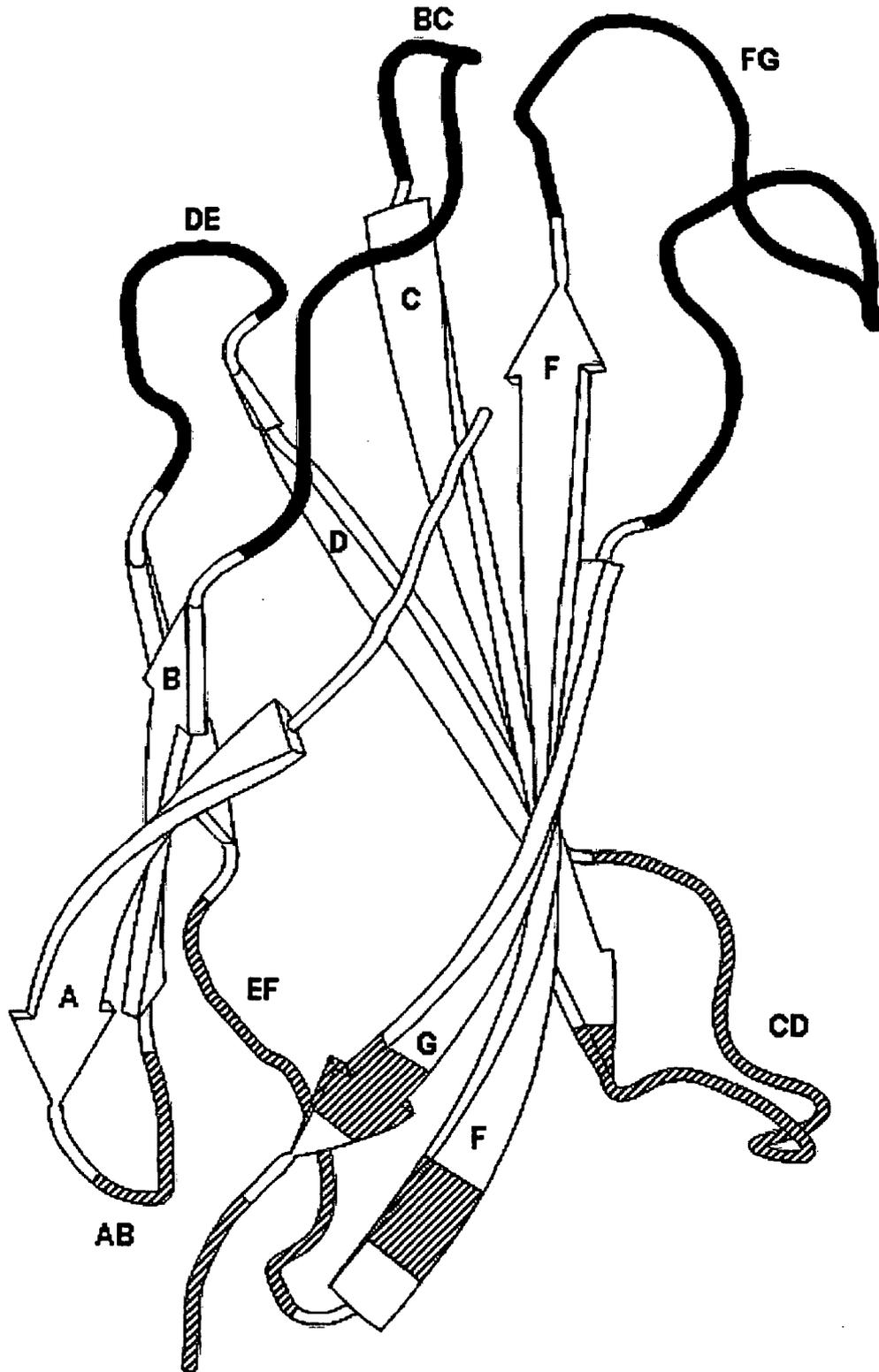
35

40

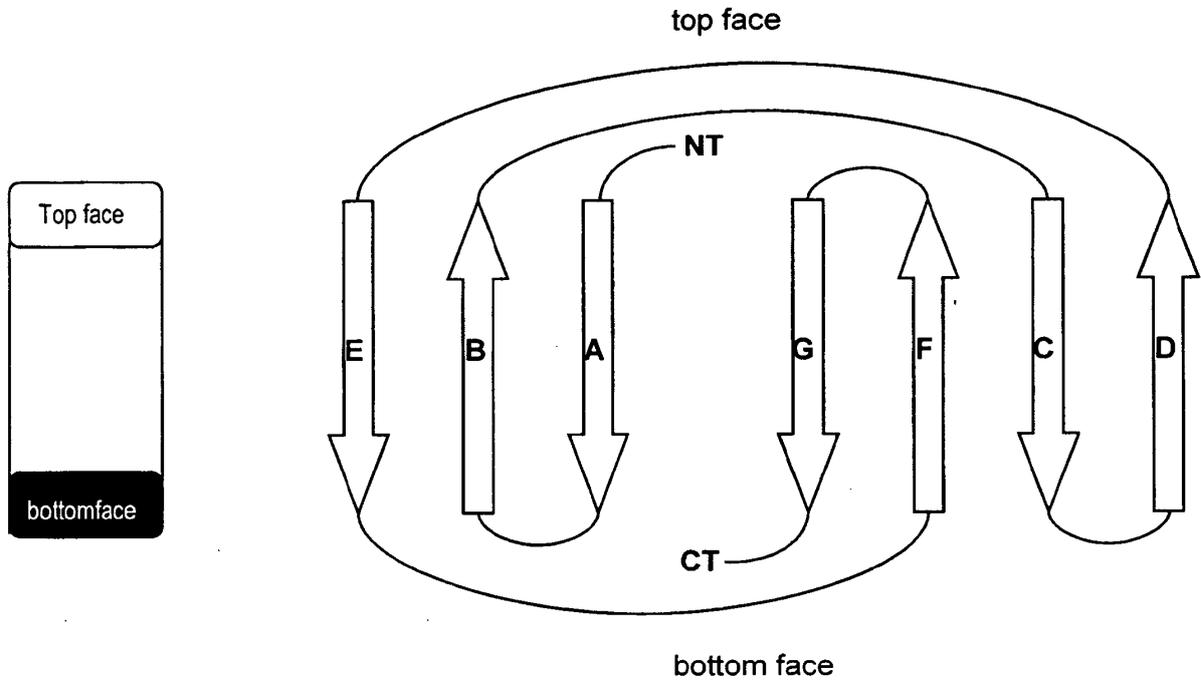
45

50

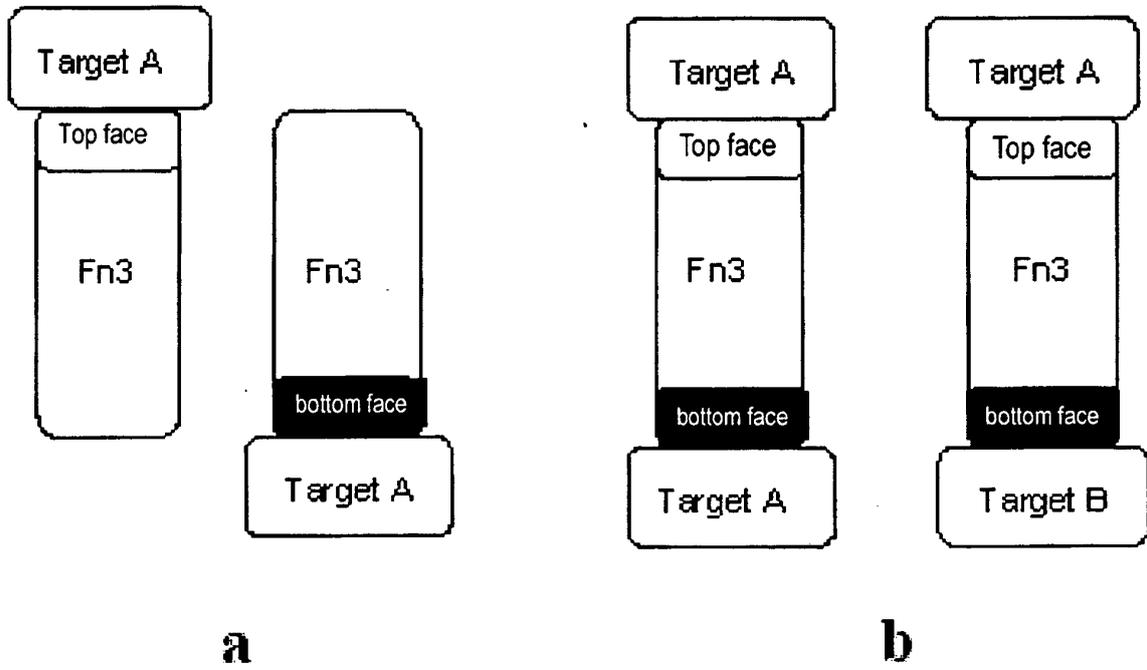
55



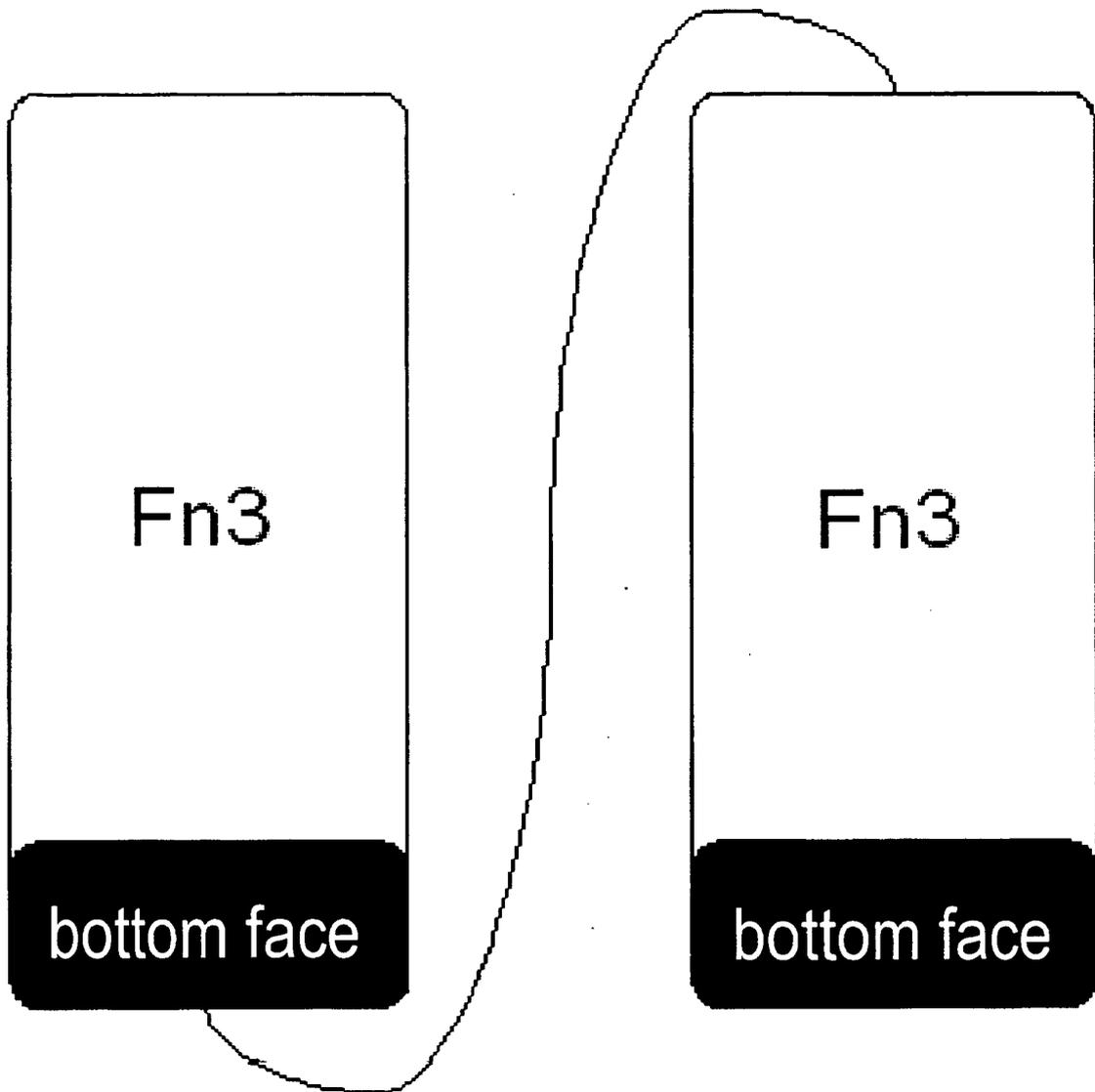
*Fig. 1*



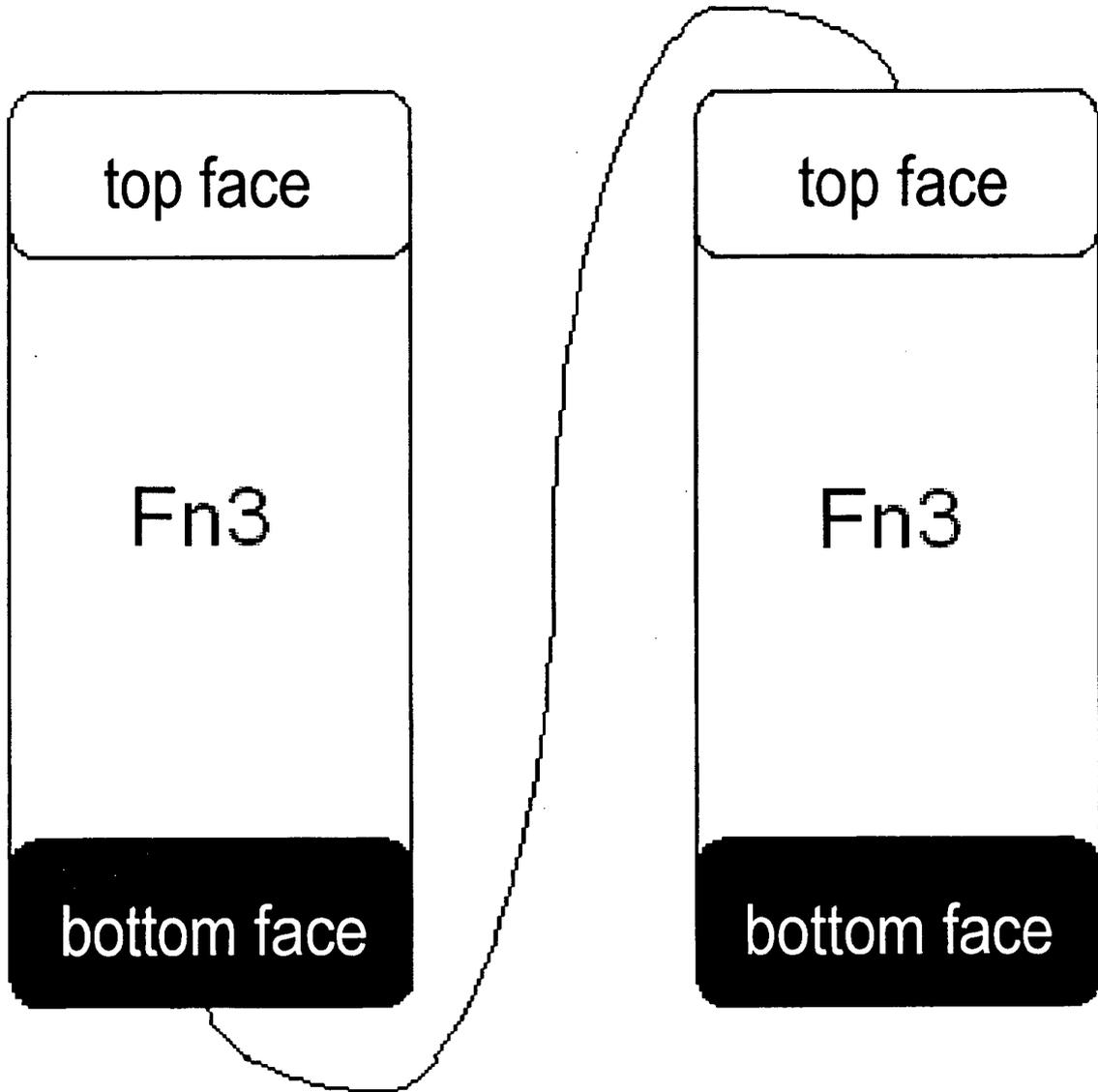
*Fig. 2*



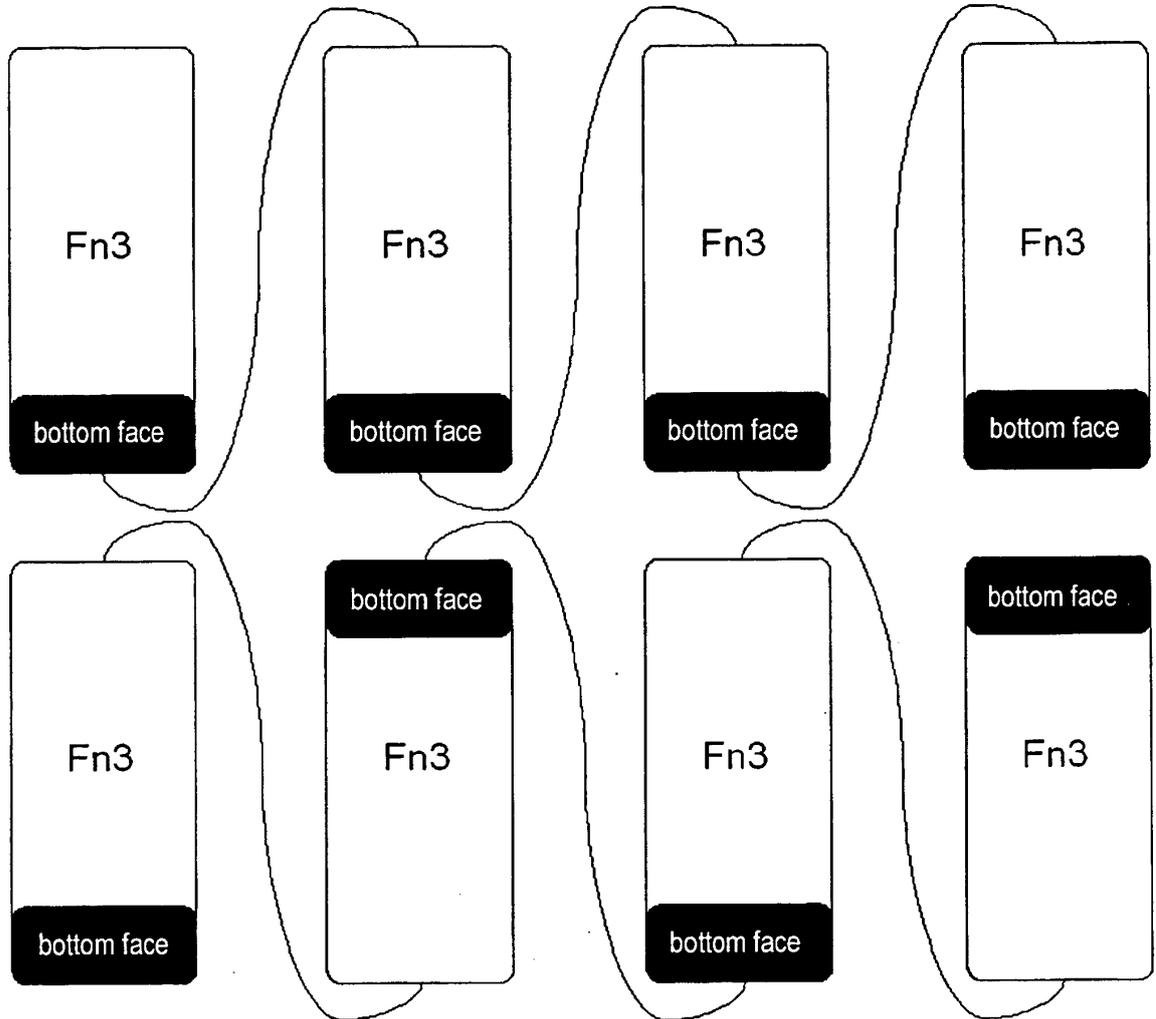
*Fig. 3*



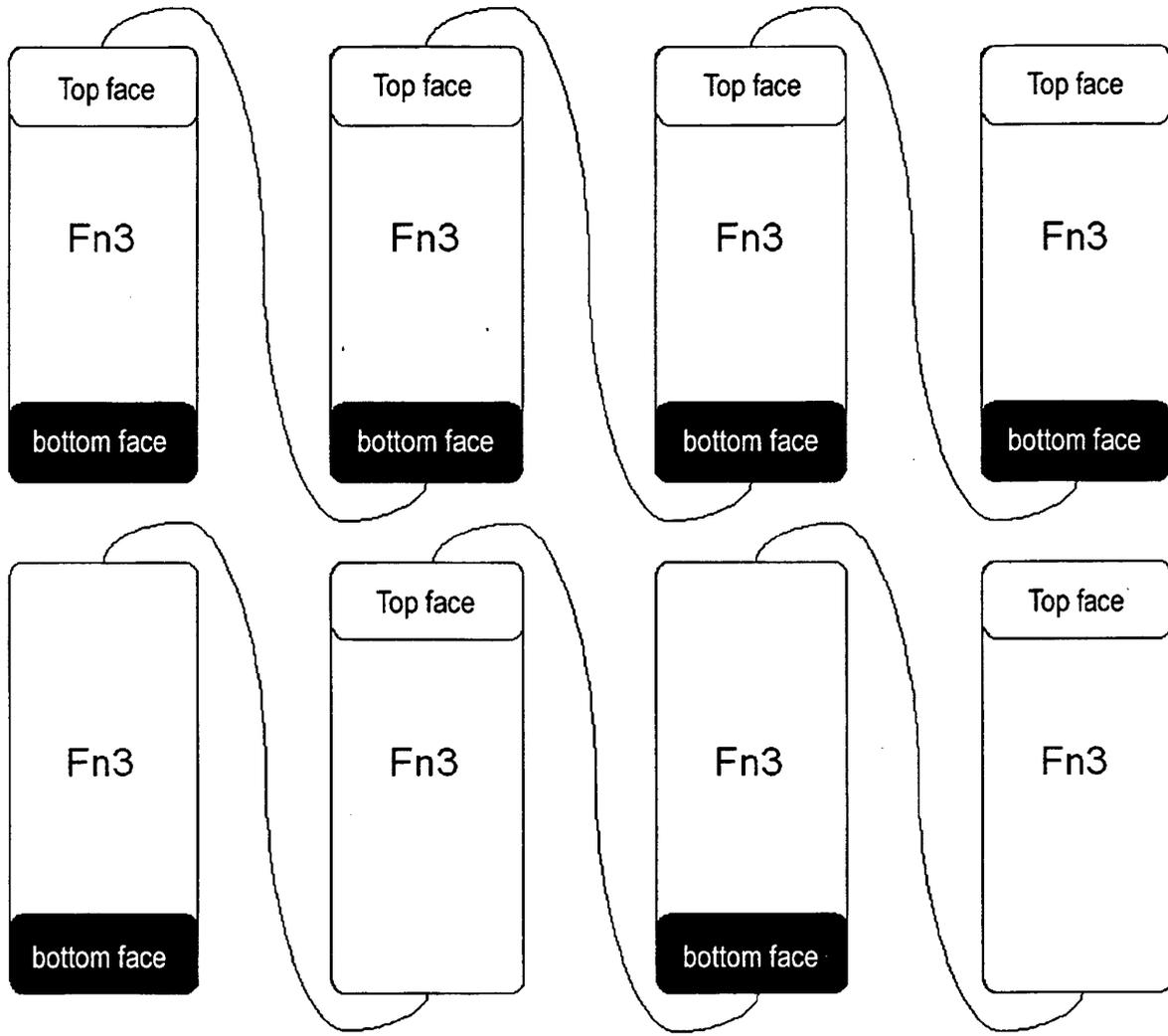
*Fig. 4a*



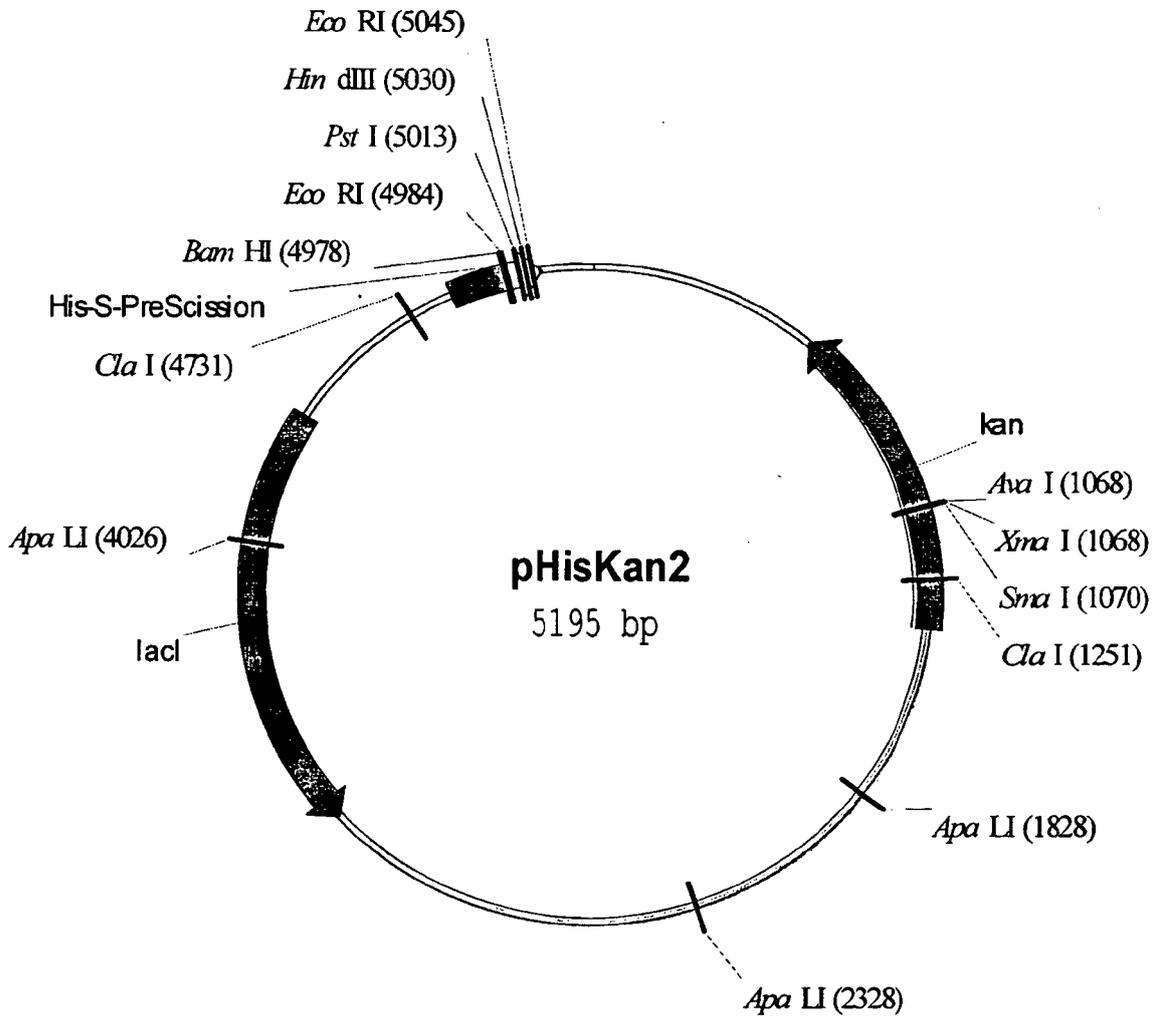
*Fig. 4b*



*Fig. 5a*



*Fig. 5b*



*Fig. 6*

SEQ ID 51 96  
 7 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G K T E G H R H S H E F T V P G S K S T A T I S G L K H G D D Y T I T V Z A V T G R G D S P A S S K P I S I M Y R M E K  
 8 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G R I D A K S T R K E F T V P G S K S T A T I G E L K R G R D Y T I T V Z A V T G R G D S P A S S K P I S I M Y R P E K  
 9 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G Q L D K K H D A E F T V P G S K S T A T I T R L K R G R D Y T I T V Z A V T G R G D S P A S S K P I S I M Y R F E R  
 10 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G K T G H K S S D H E F T V P G S K S T A T I G G M K G V D Y T I T V Z A V T G R G D S P A S S K P I S I M Y S P E R  
 11 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G K K D R A S H L K K E F T V P G S K S T A T I G H I K G V D Y T I T V Z A V T G R G D S P A S S K P I S I M Y S G H P  
 12 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G R T K G A S K L K E F T V P G S K S T A T I P G L K Q G E D Y T I T V Z A V T G R G D S P A S S K P I S I M Y M F E R  
 13 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G T S V D D R K L R E F T V P G S K S T A T I S R L K R G R D Y T I T V Z A V T G R G D S P A S S K P I S I M Y R A E N  
 14 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G V L S S T K H L K E F T V P G S K S T A T I S G L K L G R D Y T I T V Z A V T G R G D S P A S S K P I S I M Y R M E S  
 15 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G S R A S H K L T E F T V P G S K S T A T I S D V K T G R D Y T I T V Z A V T G R G D S P A S S K P I S I M Y R T E H  
 16 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G I G S E K S F V E F T V P G S K S T A T I R G L K R G R D Y T I T V Z A V T G R G D S P A S S K P I S I M Y T R A E T  
 17 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G Q R V G K T K V H E F T V P G S K S T A T I R G L K R G R D Y T I T V Z A V T G R G D S P A S S K P I S I M Y T P E T  
 18 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G H T A K T R H H E F T V P G S K S T A T I C K S R R G S D Y T I T V Z A V T G R G D S P A S S K P I S I M Y Q A E T  
 19 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G Y G A A M R R A H E F T V P G S K S T A T I S G L K R G R D Y T I T V Z A V T G R G D S P A S S K P I S I M Y R H E L  
 20 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G K I G G K P V R E F T V P G S K S T A T I L G L K P G L D Y T I T V Z A V T G R G D S P A S S K P I S I M Y I H E Q  
 21 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G D S R H E P R A H E F T V P G S K S T A T I T C C K R G H D Y T I T V Z A V T G R G D S P A S S K P I S I M Y R Y E H  
 22 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G K S H G H Q K H E F T V P G S K S T A T I S D Y R Q G G D Y T I T V Z A V T G R G D S P A S S K P I S I M Y K G E L  
 23 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G V M G D T K K Y K E F T V P G S K S T A T I E R I K L G R D Y T I T V Z A V T G R G D S P A S S K P I S I M Y R K E H  
 24 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G E A S K M H R Q H E F T V P G S K S T A T I S R I K R G T D Y T I T V Z A V T G R G D S P A S S K P I S I M Y S P E S  
 25 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G E K P R K Y R S S E F T V P G S K S T A T I R H S K H G K D Y T I T V Z A V T G R G D S P A S S K P I S I M Y M R E I  
 26 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G K T A R K Y H M Q E F T V P G S K S T A T I S G I K R G T D Y T I T V Z A V T G R G D S P A S S K P I S I M Y M S E H  
 27 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G L T K R S L I S E F T V P G S K S T A T I I G L K A G K D Y T I T V Z A V T G R G D S P A S S K P I S I M Y K T E N  
 28 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G K V K A K A P L V E F T V P G S K S T A T I S S L K T G K D Y T I T V Z A V T G R G D S P A S S K P I S I M Y R Y E G  
 29 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G D R A K M T R I K E F T V P G S K S T A T I S G L K R G R D Y T I T V Z A V T G R G D S P A S S K P I S I M Y R L E G  
 30 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G I P A K K H T H E F T V P G S K S T A T I T G L K S G V D Y T I T V Z A V T G R G D S P A S S K P I S I M Y R T E T  
 31 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G E V H G H T A H E F T V P G S K S T A T I V R L K R G K D Y T I T V Z A V T G R G D S P A S S K P I S I M Y C P E R  
 32 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G M I S G D K R R R E F T V P G S K S T A T I D R L K L G R D Y T I T V Z A V T G R G D S P A S S K P I S I M Y R L E S  
 33 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G V M H D K H P K K E F T V P G S K S T A T I S G L K R G R D Y T I T V Z A V T G R G D S P A S S K P I S I M Y K S E A  
 34 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G V A G K T K P R S E F T V P G S K S T A T I P H L K L G H D Y T I T V Z A V T G R G D S P A S S K P I S I M Y R A E H  
 35 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G K N G R H N L D H E F T V P G S K S T A T I S G L K R G V D Y T I T V Z A V T G R G D S P A S S K P I S I M Y H N E N  
 36 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G N I V A A M A L P E F T V P G S K S T A T I T G L M S G I D Y T I T V Z A V T G R G D S P A S S K P I S I M Y S T E Y  
 37 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G K L S O P T K R R E F T V P G S K S T A T I S R L K P E L D Y T I T V Z A V T G R G D S P A S S K P I S I M Y H Y R L  
 38 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G K K S H E S A A K E F T V P G S K S T A T I T R L K S G T D Y T I T V Z A V T G R G D S P A S S K P I S I M Y K P E Y  
 39 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G K K H K H T V R E F T V P G S K S T A T I A G K S L G D Y T I T V Z A V T G R G D S P A S S K P I S I M Y T S E Y  
 40 VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G R P G H M S K V H E F T V P G S K S T A T I D G A K K G H D Y T I T V Z A V T G R G D S P A S S K P I S I M Y I M E H  
 41 V S E I P K D L E V V A A T P T S L L I S W D A P A V T V R Y Y R I T Y G Q T M N G Q S N V E F T V P G S K S T A T I S P S K L G D Y T I T V Z A V T G R G D S P A S S K P I S I M L S E T  
 W T V S D V P R D L E V V A A T P T S L L I S W D A P A V T V R Y Y R I T Y G E T G G E S P V Q E F T V P G S K S T A T I S G L K P G V D Y T I T V Z A V T G R G D S P A S S K P I S I M Y R T E I  
 42 -Cons VSDVPRDLEV VAA TPT SLL I S W D A P A V T V R Y Y R I T Y G K T G G S K V H E F T V P G S K S T A T I S G L K R G R D Y T I T V Z A V T G R G D S P A S S K P I S I M Y R P E H

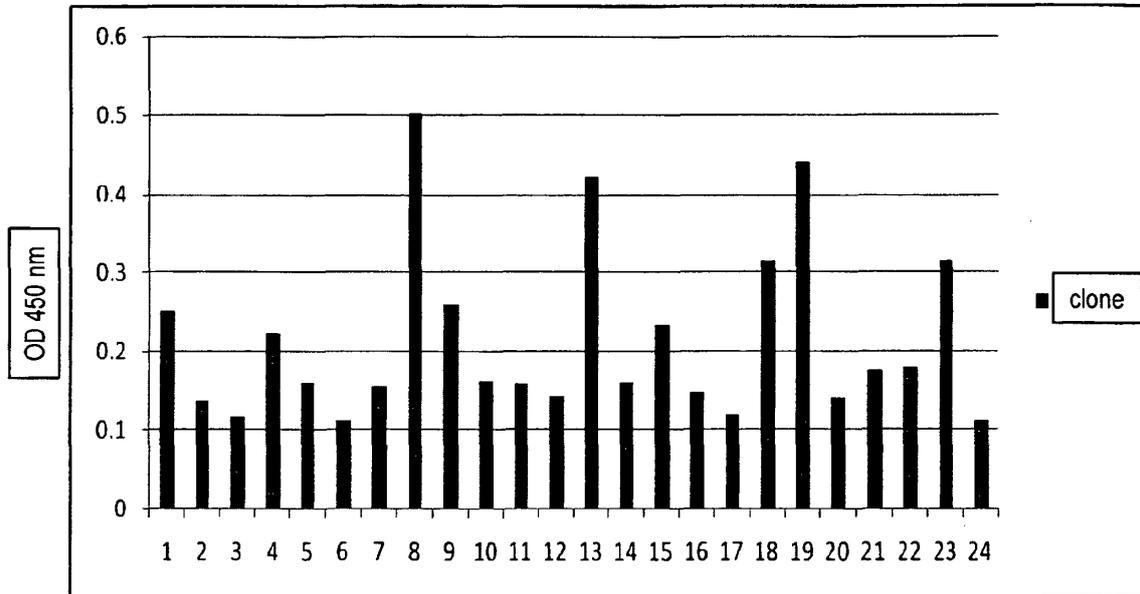
Fig. 7

SBQ ID  
43 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGE. E AYRSEQEFT VPGSKSTATI SSIYQVYDT ITVAVTVGRG ESPASSKPIS INYEGSE  
44 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGE. S SLGSE.QEFT VPGSKSTATI SBVAPYVDT ITVAVTVGRG ESPASSKPIS INYEGPG  
45 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGE. Q A.OHEQEFT VPDKSTATI SAHRSYVDT ITVAVTVGRG ESPASSKPIS INYITGE  
46 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGEYR SYRSEQEFT VPGSKSTATI SRGYRVDYDT ITVAVTVGRG ESPASSKPIS INYSYRI  
47 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGEER GYRSEQEFT VPGSKSTATI SESRYVDT ITVAVTVGRG ESPASSKPIS INYPHYSY  
48 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGE. Q SGLSEQEFT VPGSKSTATI SSLRYVDT ITVAVTVGRG ESPASSKPIS INYAYEY  
49 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGE. R GSEARQEFT VPGSKSTATI SOAYIGVDT ITVAVTVGRG ESPASSKPIS INYASIAS  
50 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGE. . SLYEQEFT VPGSKSTATI SASIAYVDT ITVAVTVGRG ESPASSKPIS INYSYHL  
51 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGESY RRGQEQEFT VPGSKSTATI SPSQSGVDT ITVAVTVGRG ESPASSKPIS INYASIAS  
52 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGESS RSP.RQEFT VPGSKSTATI SRAEYRVDYDT ITVAVTVGRG ESPASSKPIS INYLRQ  
53 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGESS PEY.RQEFT VPGSKSTATI SLEEYVDT ITVAVTVGRG EPTSSKPIS INYQYRY  
54 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGESL RSR.EQEFT VPGSKSTATI SGRQESVDT ITVAVTVGRG ESPASSKPIS INYQRY  
55 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGEGQ GAG.ASQEFT VPGSKSTATI SGAEPYVDT ITVAVTVGRG ESPASSKPIS INYGPYRY  
56 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGE. Y LYH.HQEFT VPGSKSTATI SYRSAPVDT ITVAVTVGRG ESPASSKPIS INYRHER  
57 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGES LLY.HSQEFT VPGSKSTATI SYGPAVDT ITVAVTVGRG ESPASSKPIS INYRRRY  
58 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGE. . SHGRSQEFT VPGSKSTATI SSSSRVDT ITVAVTVGRG ESPASSKPIS INYGRY  
59 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGE. . YGYSRSEQEFT VPGSKSTATI SYSSPRVDT ITVAVTVGRG ESPASSKPIS INYGSRY  
60 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGERY YAYVQEQEFT VPGSKSTATI SRSSPRVDT ITVAVTVGRG ESPASSKPIS INYPRRY  
61 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGE. P RSEEGEQEFT VPGSKSTATI SRRSLVDT ITVAVTVGRG ESPASSKPIS INYSSR  
62 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGE. A ASSRLOEFT VPGSKSTATI SSYGLVDT ITVAVTVGRG ESPASSKPIS INYSSY  
63 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGE. R ERHQEQEFT VPGSKSTATI SRRRRVDT ITVAVTVGRG ESPASSKPIS INYEPGY  
64 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGE. R RSHREQEFT VPGSKSTATI SRRRRVDT ITVAVTVGRG ESPASSKPIS INYSSRR  
65 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGE. R LREGGQEFT VPGSKSTATI SRAGERVDT ITVAVTVGRG ESPASSKPIS INYAOYR  
66 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGEY . RSY.YQEFT VPGSKSTATI SSIYVDT ITVAVTVGRG ESPASSKPIS INYRRL  
67 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGEYX ARPE.YQEFT VPGSKSTATI SYVEPEVDT ITVAVTVGRG ESPASSKPIS INYRSYX  
68 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGEYX RSSL.PQEFT VPGSKSTATI SSIYVDT ITVAVTVGRG ESPASSKPIS INYRSYX  
69 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGEYX SSI.YQEFT VPGSKSTATI SYPSERVDT ITVAVTVGRG ESPASSKPIS INYQSSA  
70 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGE. S HQGLSQEFT VPGSKSTATI SPSGSSVDT ITVAVTVGRG ESPASSKPIS INYRYR  
71 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGE. R GGLGQEFT VPGSKSTATI SRAIRVDT ITVAVTVGRG ESPASSKPIS INYALIE  
72 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGE. R G.GYSQEFT VPGSKSTATI SRRYQVDT ITVAVTVGRG ESPASSKPIS INYQSY  
73 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGE. R .GIPQEFT VPGSKSTATI SSIYVDT ITVAVTVGRG ESPASSKPIS INYRYE  
74 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGEYX PGYQEQEFT VPGSKSTATI SSIYVDT ITVAVTVGRG ESPASSKPIS INYRYE  
75 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGEYX G5..OAEFT VPGSKSTATI SREYGVDT ITVAVTVGRG ESPASSKPIS INYALGE  
76 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGESS GR..YRQEFT VPGSKSTATI SRYPHVDT ITVAVTVGRG ESPASSKPIS INYAKVEY  
77 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGEYX RR..ARQEFT VPGSKSTATI SRIEPLVDT ITVAVTVGRG ESPASSKPIS INYYSY  
78 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGEOR AR..SQEFT VPGSKSTATI SEORRVDYDT ITVAVTVGRG ESPASSKPIS INYRALL  
79 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGERR RR..YQEFT VPGSKSTATI SRRSRVDT ITVAVTVGRG ESPASSKPIS INYLYPY  
80 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGERS R5..YQEFT VPGSKSTATI SSSHRVDT ITVAVTVGRG ESPASSKPIS INYRSYL  
81 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGESH HY..GQEFT VPGSKSTATI SLLGARVDT ITVAVTVGRG ESPASSKPIS INYEGLY  
83 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGEAH EY..YQEFT VPGSKSTATI SRRSSVDT ITVAVTVGRG ESPASSKPIS INYGRY  
84 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGEAH Y5..GQEFT VPGSKSTATI SRAEYVDT ITVAVTVGRG ESPASSKPIS INYERRY  
85 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGELS R5..GQEFT VPGSKSTATI SSGYGVDT ITVAVTVGRG ESPASSKPIS INYEQSY  
86 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGELP SQ..GQEFT VPGSKSTATI SYRRELVDYDT ITVAVTVGRG ESPASSKPIS INYGSY  
87 VSDVPRDLEV VAAPTSILLI SWDAPAVTVR YRITIGEAY GE..SQEFT VPGSKSTATI SOSPEYVDT ITVAVTVGRG ESPASSKPIS INYASEY

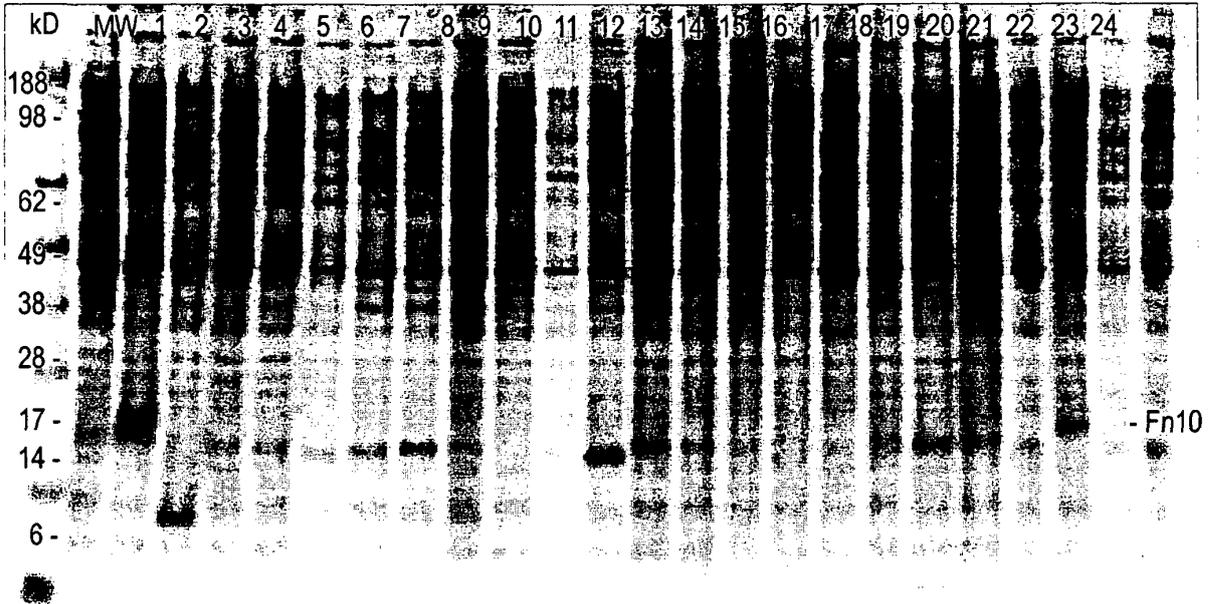
Fig. 8

SEQ ID  
88 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGESS SS..RROEFT VPGSKSTATI SGEESYVDYIT ITVYAVTGRG ESPASSKPTIS INYAERB  
89 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGEGR HP..SYOEFT VPGSKSTATI SPYSRVDYIT ITVYAVTGRG ESPASSKPTIS INYSRER  
90 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGEGE RP...YOEFT VPGSKSTATI SYAHYSVDYIT ITVYAVTGRG ESPASSKPTIS INYRERS  
91 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGEYO R...SROEFT VPGSKSTATI SSGYGVYDXT ITVYAVTGRG ESPASSKPTIS INYRSLR  
92 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGEYE R...YRQEFT VPGSKSTATI SSQRVVDYIT ITVYAVTGRG ESPASSKPTIS INYGRFA  
93 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGEYP R...EYOEFT VPGSKSTATI SRYGXHVDYIT ITVYAVTGRG ESPASSKPTIS INYSGYS  
94 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGEYE H...YYOEFT VPGSKSTATI SEYAGGVDYIT ITVYAVTGRG ESPASSKPTIS INYSESE  
95 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGEYE Y...AROEFT VPGSKSTATI SRLYRRVDYIT ITVYAVTGRG ESPASSKPTIS INYRRA  
96 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGEYH Y...SEOEFT VPGSKSTATI SYEPRVDYIT ITVYAVTGRG ESPASSKPTIS INYHBR  
97 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGEOR Y...LROEFT VPGSKSTATI SRSRARDYIT ITVYAVTGRG ESPASSKPTIS INYFSA  
98 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGERS S...RSOEFT VPGSKSTATI SHSRPVDYIT ITVYAVTGRG ESPASSKPTIS INYOYR  
99 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGESQ S...HSOEFT VPGSKSTATI SHSRPVDYIT ITVYAVTGRG ESPASSKPTIS INYPRSE  
100 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGEEE E...HPOEFT VPGSKSTATI SPQORGVDYIT ITVYAVTGRG ESPASSKPTIS INYYSY  
101 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGEEES S...RPOEFT VPGSKSTATI SQARARVDYIT ITVYAVTGRG ESPASSKPTIS INYRRYR  
102 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGEEY A...LEOEFT VPGSKSTATI SRQAEVDYIT ITVYAVTGRG ESPASSKPTIS INYASY  
103 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGESR E...GGOEFT VPGSKSTATI SRYQSEVDYIT ITVYAVTGRG ESPASSKPTIS INYARBR  
104 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGEGQ Q...GGOEFT VPGSKSTATI BAYRQVDYIT ITVYAVTGRG ESPASSKPTIS INYYSY  
105 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGEBR R...YGOEFT VPGSKSTATI SELLSEVDYIT ITVYAVTGRG ESPASSKPTIS INYSLRY  
106 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGESC Y...RYOEFT VPGSKSTATI SGYREHVDYIT ITVYAVTGRG ESPASSKPTIS INYQESY  
107 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGESC R...RGOEFT VPGSKSTATI SPSLEVDYIT ITVYAVTGRG ESPASSKPTIS INYRSY  
108 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGEP R...RYOEFT VPGSKSTATI SPGGSVDYIT ITVYAVTGRG ESPASSKPTIS INYRGY  
109 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGESS RG..A.OEFT VPGSKSTATI SGLPPPVDYIT ITVYAVTGRG ESPASSKPTIS INYRSQH  
110 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGESS GYAERGOEFT VPGSKSTATI SPGARVDYIT ITVYAVTGRG ESPASSKPTIS INYSSLG  
111 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGESE GGSRSOEFT VPGSKSTATI SRYQFVDYIT ITVYAVTGRG ESPASSKPTIS INYGSYP  
112 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGEOY YHQRRQOFT VPGSKSTATI SRYGGSVDYIT ITVYAVTGRG ESPASSKPTIS INYAYG  
113 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGERR GQYSSOFT VPGSKSTATI SPPEGSVDYIT ITVYAVTGRG ESPASSKPTIS INYRSE  
114 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGEOR YSYGQOFT VPGSKSTATI SSGRSSVDYIT ITVYAVTGRG ESPASSKPTIS INYRBSY  
115 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGERP ASYRAAOFT VPGSKSTATI SSYSYSVDYIT ITVYAVTGRG ESPASSKPTIS INYRBSG  
116 VSDVPRDLEV VAAATPTSILLI SWDAPAVTVR YRITYGEPQ ASHRPYOFT VPGSKSTATI SRSRQSVYIT ITVYAVTGRG ESPASSKPTIS INYLRBL

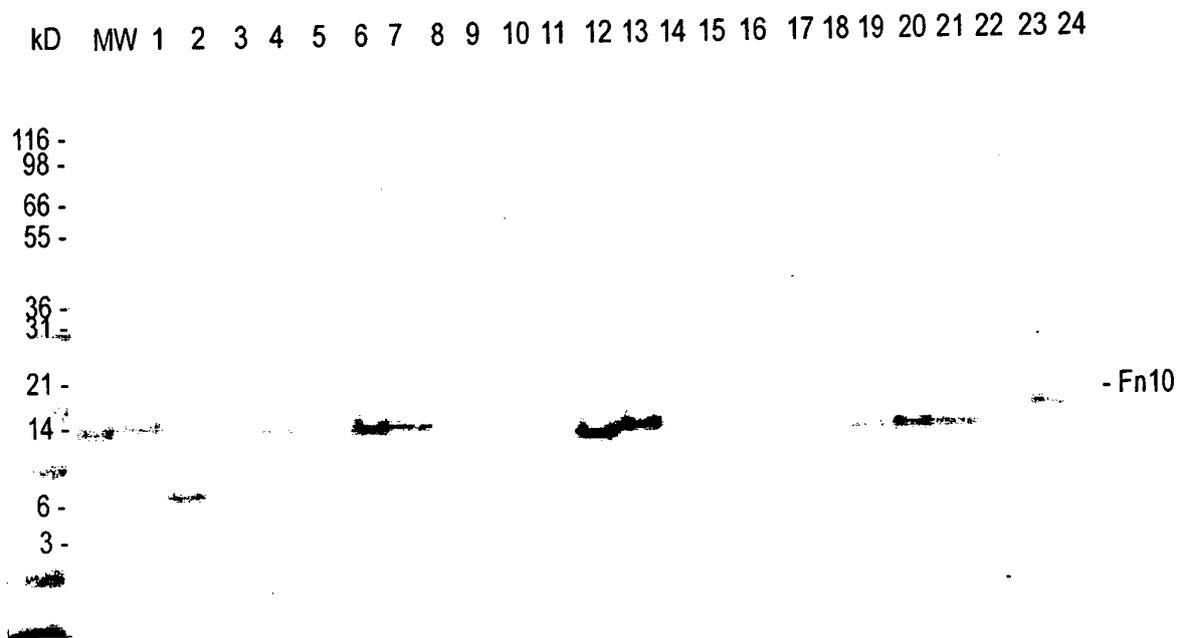
Fig. 8 cont.



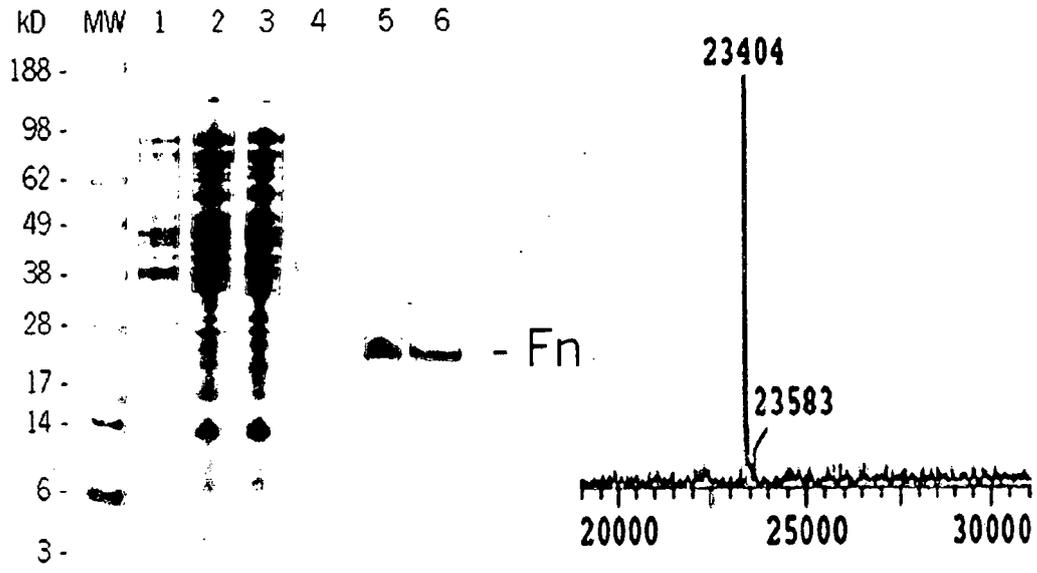
*Fig. 9*



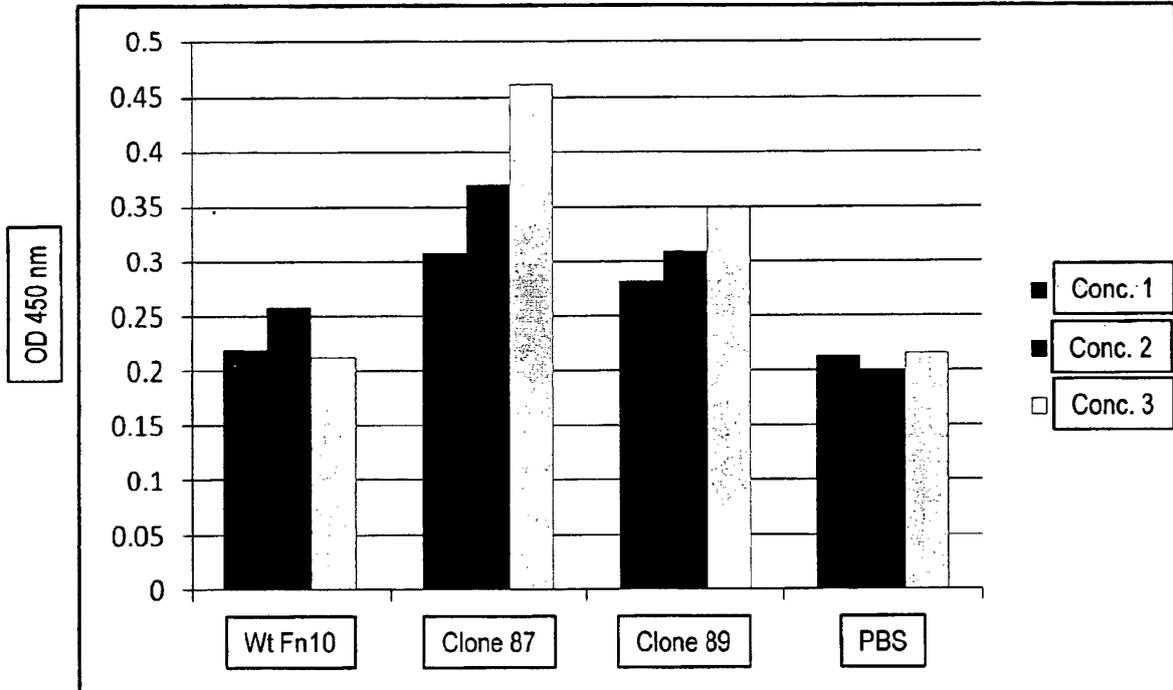
*Fig. 10*



*Fig. 11*

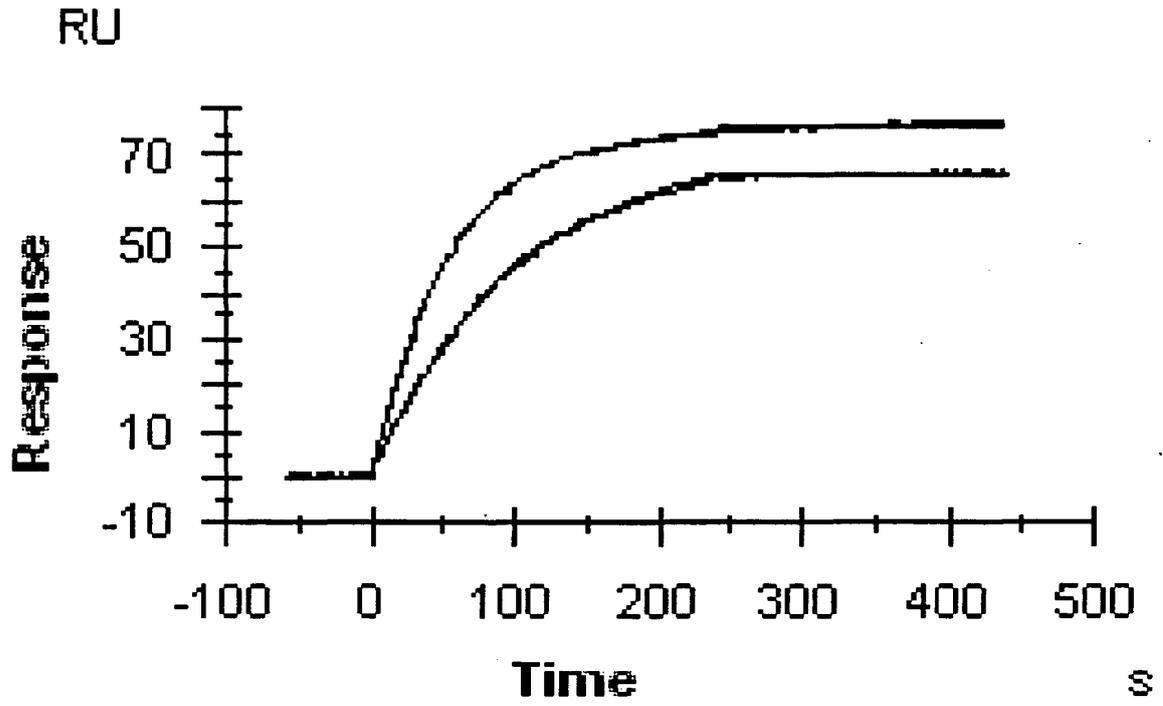


*Fig. 12*



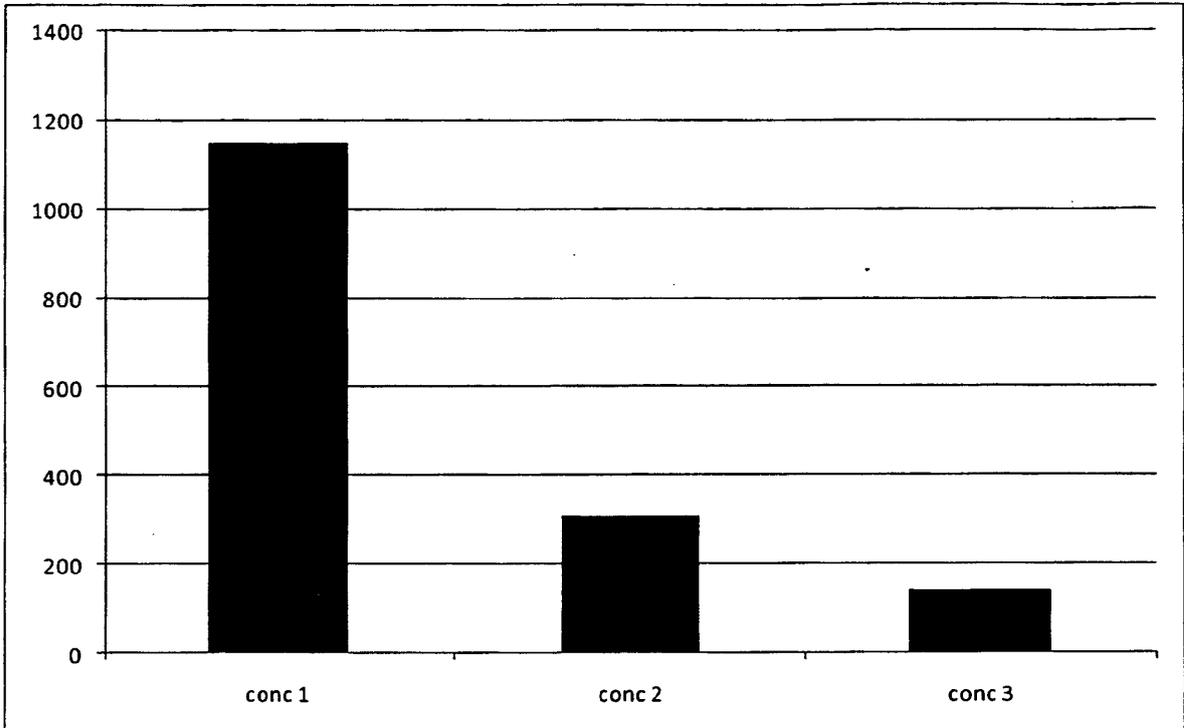
*Fig. 13*

*VEGFR2 binding of clone 87*



*Fig. 14*

*HSA binding of clone 87*



*Fig. 15*

## REFERENCES CITED IN THE DESCRIPTION

*This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.*

## Patent documents cited in the description

- US 61050142 A [0001]
- US 6673901 B [0021] [0150]
- WO 03104418 A [0021]
- US 20070082365 A [0021]
- WO 07042289 A [0030]
- US 5759808 A [0030]
- US 7195880 B [0043] [0098] [0099] [0111]
- US 6951725 B [0043] [0099] [0111]
- US 7078197 B [0043] [0099] [0111]
- US 7022479 B [0043] [0099] [0111]
- US 5922545 A [0043]
- US 5830721 A [0043]
- US 5605793 A [0043]
- US 5830650 A [0043]
- US 6194550 B [0043] [0098] [0099] [0111]
- US 6699658 B [0043] [0097] [0099] [0111]
- US 7063943 B [0043] [0097] [0099] [0111]
- US 5866344 A [0043]
- WO 06023144 A [0043] [0099] [0111]
- WO 2005056764 A [0057] [0074] [0147]
- US 20060286603 A [0062]
- WO 04041862 A2 [0062]
- US 5428130 A [0066]
- WO 9315199 A [0067]
- WO 9315200 A [0067]
- EP 413622 A [0067]
- EP 399666 A [0067]
- WO 2001077137 A [0068]
- WO 200306007 A [0068]
- US 5260203 A [0071]
- US 5455030 A [0071]
- US 4881175 A [0071]
- US 5132405 A [0071]
- US 5091513 A [0071]
- US 5476786 A [0071]
- US 5013653 A [0071]
- US 5258498 A [0071]
- US 5482858 A [0071]
- US 7045337 B [0074] [0095]
- US 7083970 B [0074] [0095]
- US 6927042 B [0074]
- EP 0154316 A, Nishimura [0074]
- EP 0401384 A, Ishikawa [0074]
- DE 19628705 [0082]
- DE 10129369 [0082]
- EP 0202928 W [0082]
- US 20070134197 A [0082]
- US 20060258607 A [0082]
- US 20060217293 A [0082]
- US 20060100176 A [0082]
- US 20060052342 A [0082]
- EP 1176195 A, Hang [0084]
- WO 03035835 A, Presta [0084]
- EP 1297172 B1 [0084]
- US 5866344 B [0097] [0099] [0111]
- US 5922545 B [0099] [0111]
- US 5830721 B [0099] [0111]
- US 5605793 B [0099] [0111]
- US 5830650 B [0099] [0111]
- US 4704362 A, Itakura [0103]
- US 5741957 A, Deboer [0107]
- US 5304489 A, Rosen [0107]
- US 5849992 A, Meade [0107]
- US 5225539 A [0112]
- US 6180370 B [0114]
- US 5693762 B [0114]
- US 5693761 B [0114]
- US 5585089 B [0114]
- US 7022500 B [0114]
- US 6548640 B [0114]
- US 6982321 B [0114]
- US 6407213 B [0114]
- US 6639055 B [0114]
- US 5859205 B [0114]
- US 6632927 B [0114]
- US 5399163 A [0134]
- US 5383851 A [0134]
- US 5312335 A [0134]
- US 5064413 A [0134]
- US 4941880 A [0134]
- US 4790824 A [0134]
- US 4596556 A [0134]
- US 4487603 A [0134]
- US 4486194 A [0134]
- US 4447233 A [0134]
- US 4447224 A [0134]
- US 4439196 A [0134]
- US 4475196 A [0134]
- US 4522811 A [0135]
- US 5374548 A [0135]
- US 5399331 A [0135]
- US 5416016 A, Low [0135]
- US PN6524583 A [0147]
- US 7153661 B [0150]
- US 7119171 B [0150]
- US 7078490 B [0150]
- US 6703199 B [0150]
- US 6462189 B [0150]

## Non-patent literature cited in the description

- JOSEFINA et al. *Nature Biotechnology*, 1997, vol. 15, 159-163 [0004]
- WU et al. *Nature Biotechnology*, 2007, vol. 25, 1290-1297 [0004]
- BORK ; DOOLITTLE. *Proc. Natl. Acad. Sci. U.S.A.*, 1992, vol. 89, 8990 [0021]
- BORK et al. *Nature Biotech.*, 1997, vol. 15, 553 [0021]
- MEINKE et al. *J. Bacteriol.*, 1993, vol. 175, 1910 [0021]
- WATANABE et al. *J. Biol. Chem.*, 1990, vol. 265, 15659 [0021]
- KABAT, E.A. et al. Sequences of Proteins of Immunological Interest. U.S. Department of Health and Human Services, 1991 [0028]
- CHOTHIA, C. et al. *J. Mol. Biol.*, 1987, vol. 196, 901-917 [0028]
- BIRD et al. *Science*, 1988, vol. 242, 423-426 [0029]
- HUSTON et al. *Proc. Natl. Acad. Sci. U.S.A.*, 1988, vol. 85, 5879-5883 [0029]
- STIJLEMANS, B. et al. *Engineered libraries of camelid antibodies and antibody fragments are commercially available*, 2004 [0030]
- KARPOVSKY et al. *J. Exp. Med.*, 1984, vol. 160, 1686 [0064] [0070]
- LIU, MA et al. *Proc. Natl. Acad. Sci. U.S.A.*, 1985, vol. 82, 8648 [0064] [0070]
- PAULUS. *Behring Ins. Mitt. No. 78*, 1985, 118-132 [0064] [0070]
- BRENNAN et al. *Science*, 1985, vol. 229, 81-83 [0064] [0070]
- GLENNIE et al. *J. Immunol.*, 1987, vol. 139, 2367-2375 [0064] [0070]
- WEINTRAUB, B. Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques. *The Endocrine Society*, March 1986 [0075]
- SAITO, G. et al. *Adv. Drug Deliv. Rev.*, 2003, vol. 55, 199-215 [0077]
- TRAIL, P.A. et al. *Cancer Immunol. Immunother.*, 2003, vol. 52, 328-337 [0077]
- PAYNE, G. *Cancer Cell*, 2003, vol. 3, 207-212 [0077]
- ALLEN, T.M. *Nat. Rev. Cancer*, 2002, vol. 2, 750-763 [0077]
- PASTAN, I. ; KREITMAN, R. J. *Curr. Opin. Investig. Drugs*, 2002, vol. 3, 1089-1091 [0077]
- SENTER, P.D. ; SPRINGER, C.J. *Adv. Drug Deliv. Rev.*, 2001, vol. 53, 247-264 [0077]
- Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy. ARNON et al. *Monoclonal Antibodies And Cancer Therapy*. Alan R. Liss, Inc, 1985, 243-56 [0080]
- Antibodies For Drug Delivery. HELLSTROM et al. *Controlled Drug Delivery*. Marcel Dekker, Inc, 1987, 623-53 [0080]
- Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review. THORPE et al. *Monoclonal Antibodies '84: Biological And Clinical Applications*. 1985, 475-506 [0080]
- Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy. *Monoclonal Antibodies For Cancer Detection And Therapy*. Academic Press, 1985, 303-16 [0080]
- THORPE et al. The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates. *Immunol. Rev.*, 1982, vol. 62, 119-58 [0080]
- BORMAN. *Chem. & Eng. News*, 2006, vol. 84 (36), 13-22 [0083]
- BORMAN. *Chem. & Eng. News*, 2007, vol. 85, 19-20 [0083]
- SHIELDS, R.L. et al. *J. Biol. Chem.*, 2002, vol. 277, 26733-26740 [0084]
- CARTER et al. *Nucleic Acids Res.*, 1985, vol. 13, 4431-4443 [0087]
- KUNKEL et al. *Proc. Natl. Acad. Sci. U.S.A.*, 1987, vol. 82, 488 [0087]
- HIGUCHI. *PCR Protocols*. Academic Press, 1990, 177-183 [0088]
- VALLETTE et al. *Nuc. Acids Res.*, 1989, vol. 17, 723-733 [0088]
- WELLS et al. *Gene*, 1985, vol. 34, 315-323 [0089]
- WINNACKER. *From Genes to Clones*. VCH Publishers, 1987 [0106]
- QUEEN et al. *Immunol. Rev.*, 1986, vol. 89, 49 [0106]
- CO et al. *J. Immunol.*, 1992, vol. 148, 1149 [0106]
- SAMBROOK et al. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Press, 1989 [0108]
- SCOPES. *Protein Purification*. Springer-Verlag, 1982 [0109]
- KABAT et al. Sequences of Proteins of Immunological Interest. U.S. Dept. of Health and Human Services, 1983 [0112]
- CHOTHIA et al. *J. Mol. Biol.*, 1987, vol. 196, 901-917 [0112]
- MACCALLUM et al. *J. Mol. Biol.*, 1996, vol. 262, 732-745 [0112]
- BERGE, S.M. et al. *J. Pharm. Sci.*, 1977, vol. 66, 1-19 [0118]
- Sustained and Controlled Release Drug Delivery Systems. Marcel Dekker, Inc, 1978 [0119]
- STREJAN et al. *J. Neuroimmunol.*, 1984, vol. 7, 27 [0120]
- V.V. RANADE. *J. Clin. Pharmacol.*, 1989, vol. 29, 685 [0135]
- UMEZAWA et al. *Biochem. Biophys. Res. Commun.*, 1988, vol. 153, 1038 [0135]
- P.G. BLOEMAN et al. *FEBS Lett.*, 1995, vol. 357, 140 [0135]
- M. OWAIS et al. *Antimicrob. Agents Chemother.*, 1995, vol. 39, 180 [0135]

## EP 2 274 331 B1

- **BRISCOE et al.** *Am. J. Physiol.*, 1995, vol. 1233, 134 [0135]
- **SCHREIER et al.** *J. Biol. Chem.*, 1994, vol. 269, 9090 [0135]
- **K. KEINANEN ; M.L. LAUKKANEN.** *FEBS Lett.*, 1994, vol. 346, 123 [0135]
- **J.J. KILLION ; I.J. FIDLER.** *Immunomethods*, 1994, vol. 4, 273 [0135]
- **CUNNINGHAM-RUNDLES C ; ZHUO Z ; GRIFFITH B ; KEENAN J.** Biological activities of polyethylene-glycol immunoglobulin conjugates. Resistance to enzymatic degradation. *J Immunol Methods*, 1992, vol. 152, 177-190 [0136]
- **LANDOR M.** Maternal-fetal transfer of immunoglobulins. *Ann Allergy Asthma Immunol*, 1995, vol. 74, 279-283 [0136]
- **MCLEOD DS et al.** *Invest Ophthalmol Vis Sci.*, February 2000, vol. 43 (2), 474-82 [0147]
- **WATANABE et al.** *Exp Dermatol.*, November 2004, vol. 13 (11), 671-81 [0147]
- **YOSHIJI H et al.** *Gut.*, September 2003, vol. 52 (9), 1347-54 [0147]
- **VERHEUL et al.** *Oncologist*, 2000, vol. 5 (1), 45-50 [0147]
- **BOLDICKE et al.** *Stem Cells*, 2001, vol. 19(1), 24-36 [0147]
- **SAMBROOK ; FRITSCH ; MANIATIS.** *Molecular Cloning.* Cold Spring Harbor Laboratory Press, 1989 [0150]
- *Antibody Engineering Protocols.* **PAUL, S.** *Methods in Molecular Biology.* Humana Pr, 1996, vol. 510 [0150]
- *Antibody Engineering: A Practical Approach.* Practical Approach Series, 1996, vol. 169 [0150]
- **HARLOW et al.** *Antibodies: A Laboratory Manual.* C.S.H.L. Press, 1999 [0150]
- *Current Protocols in Molecular Biology.* John Wiley & Sons, 1992 [0150]
- **LIPOVSEK, D. et al.** *J Mol Biol.*, 11 May 2007, vol. 368 (4), 1024-41 [0159]
- **GRÄSLUND et al.** *Nature Methods*, 2008, vol. 5, 135-146 [0192]